Prognostic and predictive factors in primary breast cancer

PROGNOSTIC AND PREDICTIVE FACTORS IN PRIMARY BREAST CANCER

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PROGNOSTIC AND PREDICTIVE FACTORS IN PRIMARY BREAST CANCER

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To all breast cancer patients who
suffered from this unpredictable disease

CONTENTS

Chapter I

INTRODUCTION

INTRODUCTION

Breast cancer is a major public health problem. Since 1975 the incidence of the disease has increased by an average of 1% per year, heightening concern among physicians and women in general. On the basis of incidence rates for 1975 through 1987 and mortality rates for 1987 in the United States[1,2], and the Netherlands[3], 10.7-12 percent of all women will be diagnosed with breast cancer and 3.5-3.9 percent will die of the disease. The impact of breast cancer is magnified because women are at a cumulative risk from their middle to later years. The incidence rates of breast cancer increase steadily during the fourth decade and become substantial before the age of 50, thus creating a long-lasting source of concern for women and a need for vigilance. After menopause the incidence rates continue to increase with age. Breast cancer is the leading cause of death among Western-European and American[2] women who are 40 to 55 years of age. In less prosperous parts of the world and in the Far East, the same pattern of increase with age is seen[4], but the absolute rates are much lower at each age. In Japan, for example, the overall incidence of breast cancer is only about one fifth of that in the United States[5]. Long-term increases in the incidence of breast cancer are being observed worldwide in both industrialized and developing countries[6,7]. The age adjusted mortality rates have been remarkably stable, despite increases in incidence which can be the result of more complete reporting of incident cases, increases of a more benign form of disease, earlier detection, or advances in treatment.

A number of variables that predict for the occurrence of breast cancer and their typical relative risks are described briefly in table I. As can be appreciated, the established risk factors for breast cancer - a family history of breast cancer, early menarche, late age at first childbirth, late age of menopause, history of benign breast disease and exposure to ionizing radiation - are generally associated with only weak or moderate elevations in risk. The exceptions occur in subgroups of these variables; for example a family history of breast cancer at a young age or a family history of bilateral disease[8,9]. An intensive search for DNA markers for familial risk is ongoing. A category of inherited breast cancers is associated with a gene on chromosome 17q, termed breast cancer 1 or BRCA1[10,11].

Table I Variables predicting the occurrence of breast cancer.

Risk factor	Relative risk
Family history (maternal)	
one first degree relative	2
first-degree relative diagnosed < 40 years	3
two first-degree relatives	4
bilateral breast cancer	4
Benign breast disease	
biopsy confirmed	2
atypical hyperplasia	4
mammographic (dysplasia/prominent ducts)	1.5
Lobular carcinoma in situ	10
Nulliparity/first child when > 30 years	2

STAGING

The clinical course of breast cancer is determined by the extent of the disease. Staging refers to procedures performed to determine groups of patients with a same extent of local, regional or distant cancer involvement. Breast cancer is staged initially on a clinical basis, according to the results of physical examination, laboratory and radiologic evaluations. This information can be useful in choosing treatments, estimating prognosis and comparing the results of different treatment programs. An internationally recognized staging system - the TNM system - has been accepted by both the Union Internationale Contre le Cancer (UICC) and the American Joint Commission on Cancer Staging and End-Results Reporting[12]. This system is based on the extent of the tumour, the involvement of lymph nodes, and the presence of metastases (table II).
Because the clinical evaluation of axillary - node involvement has considerable false positive and false negative rates, pathological (post operative) staging based on histologic evaluation of the axillary specimen provides a more accurate assessment of prognosis for the individual patient. The prognosis of breast cancer is clearly related to extent of axillary involvement, therefore, it has become conventional to categorize patients according to the number of involved nodes - that is no positive nodes (node-negative), 1 to 3 positive nodes, 4 to 9 positive nodes and 10 or more positive nodes.

HISTOLOGY OF INVASIVE BREAST CANCER

Invasive breast cancers are a histologically heterogeneous group of lesions. Most breast carcinomas are adenocarcinomas and are classified as either ductal or lobular, corresponding to the ducts and lobules of the normal breast. In addition, there are a number of less common types of invasive breast carcinomas. The various types have slightly different prognosis, but it is not known whether the response to chemotherapy differs, since patients in therapeutic trials have not been stratified according to histologic type.

Table II TNM system and clinical stage

T_x		Primary tumour cannot be assessed
T_0		No evidence of primary tumour
Tis		Carcinoma in situ: intraductal carcinoma, lobular carcinoma in situ, or Paget's disease of the nipple without tumour
T_1		Tumour ≤ 2 cm
	a	Tumour ≤ 0.5 cm
	b	Tumour > 0.5 cm, but not > 1 cm
	c	Tumour > 1 cm, but not > 2 cm
T_2		Tumour > 2 cm, but not > 5 cm
T_3		Tumour > 5 cm
T_4		Tumour of any size with direct extension to chest wall* or skin**
	a	Extension to chest wall
	b	Edema (including peau d'orange), ulceration of the skin or the breast, or satellite skin nodules confined to the same breast
	c	Both of above
	d	Inflammatory carcinoma
N_x		Regional lymph nodes cannot be assessed (e.g., previously removed)
N_0		No regional lymph node metastases
N_1		Metastasis to movable ipsilateral axillary nodes
N_2		Metastasis to ipsilateral axillary nodes fixed to an other or to other structures
N_3		Metastasis to ipsilateral internal mammary lymph nodes
M_0		No evidence of distant metastasis
M_1		Distant metastases (including metastases to ipsilateral supraclavicular lymph nodes)

Clinical Stage	
I	T_1, N_0, M_0
IIA	T_0, N_1, M_0
	T_1, N_1, M_0
	T_2, N_0, M_0
IIB	T_2, N_1, M_0
	T_3, N_0, M_0
IIIA	T_0 or T_1, N_2, M_0
	T_2, N_2, M_0
	T_3, N_1 or N_2, M_0
IIIB	T_4, any N, M_0
	Any T, N_3, M_0
IV	Any T, any N, M_1

* The chest wall includes the ribs, intercostal muscles, and serratus anterior muscle, but not the pectoral muscle.

** Dimpling of the skin, nipple retraction, or any skin changes except those listed for T_{4b} may occur in T_1, T_2 or T_3 without affecting the classification.

ADJUVANT THERAPY OF BREAST CANCER

The demonstration that adjuvant systemic treatment can prolong the disease-free interval and improve overall survival has been a major advance in the management of breast cancer. Randomized clinical trials of systemic adjuvant chemotherapy were pioneered by the Milan group[13] and the NSABP-National Surgical Adjuvant Breast and Bowel Projectgroup[14] and provided the first evidence of the benefit of cytotoxic chemotherapy in breast cancer patients with involvement of the axillary lymph nodes, particularly in younger women.
The Nolvadex Adjuvant Trial Organization[15] and the Scottish Trial[16] demonstrated a similar benefit for tamoxifen in older women. These early studies were followed by a large number of national and international multicenter cooperative clinical trials. Most of these trials had relatively simple designs, comparing multidrug regimens with no treatment or comparing tamoxifen with placebo. In addition, there have been a number of trials testing the value of combining chemotherapy and tamoxifen[17,18].
On the basis of available information a number of general conclusions on the effects of adjuvant systemic treatment can be drawn[19,20,21]. The effect on disease-free interval (DFI) has generally been larger than the effect on overall survival (OS). The effect of polychemotherapy has been larger than the effect of mono-drug therapy. Polychemotherapy has a greater effect in premenopausal patients, and tamoxifen is more effective in postmenopausal women. The administration of full doses of chemotherapy is associated with improved results. Sustained chemotherapy after six months has no additional benefit whereas several years of tamoxifen therapy has provided more advantages than a single year of use. No consistent evidence supports the addition of tamoxifen to chemotherapy in younger women; in older women, there is some evidence that the addition of chemotherapy containing anthracyclines to tamoxifen results in improved OS[17]. The benefit of tamoxifen is greater in patients with oestrogen-receptor positive tumours than in those with oestrogen-receptor negative tumours.

Because the overall effects of most individual trials particularly on survival have been small, relatively large numbers of patients are required to observe small but clinically important effects in subgroups of patients. This has led to the use of a combined analysis (meta-analysis) of all breast cancer trials, based on a worldwide collaboration of 133 randomized trials with 31.000 recurrences of disease and 24.000 deaths among 75.000 women[19]. Table III lists the reduction in the annual odds of either recurrence or death after treatment, as compared with the annual odds for the control group, as a function of therapy and age.
The reduction is an approximation of the relative risk in the rate of recurrence or death in the treated group as compared with the control group. The absolute benefit of adjuvant therapy will depend on the patients initial risk of dying from the disease. If, for example, adjuvant therapy reduces the relative risk of death by 30%, then, the absolute benefit (the additional percentage of patients alive at 10 years) will be about 4 percent for patients with stage I disease and good prognostic factors, who have a 10-20% risk of death from breast cancer and 8% for patients with stage I disease and poor prognostic factors, who have a 20-40% risk of death. If adjuvant therapy reduces the relative risk by 15%, the corresponding absolute benefit, in the latter example, will be 10-20%.

Although meta-analysis can detect small benefits of treatment, they usually do not identify differences between trials in such variables as dose intensity, drug selection and sequencing, or population differences that may be important in the outcome.

One of the most controversial aspects on the use of adjuvant therapy is its role in breast cancer patients with axillary nodes free of metastases. The rate of recurrence in this group of patients is approximately 10-40% and a series of recent trials have established that adjuvant therapy is of value for at least some of these women[22,23,24,25]. Guidelines for the use of adjuvant chemotherapy in patients with node-negative cancer have not been established. Adjuvant therapy is currently being explored in node-negative patients with unfavourably prognostic factors including steroid-receptor negative tumours.

Table III Reduction in the annual odds of recurrence or death after treatment

Type of therapy	Comparison group	Recurrence		Death	
		< 50 yr	≥ 50 yr	< 50 yr	≥ 50 yr
Polychemother.	No chemother.	36±5	24±3	24±5	13±4
Tamoxifen	No tamoxifen	12±4	29±2	6±5	20±2

* Data are from the Early Breast Cancer Trialists' Collaborative Group (17) plus-minus values are means ± SD.

METASTATIC BREAST CANCER

Metastatic breast cancer remains an incurable disease, although systemic therapy can provide effective temporary palliation for many patients. Initial combination chemotherapy for metastatic breast cancer produces response rates in the range of 50% to 70%, but only 10% to 20% of the patients achieve complete remissions[26,27,28]. For most patients, remission duration is relatively brief, and prolongation of median survival with any chemotherapy regimen is modest at best[29]. Several trials carried out over the past 15 years have shown some improvement in response rates and frequency of complete remission, but they have not demonstrated a consistent increase in the duration of response or in survival[30,31,32,33]. When patients relapse after first line chemotherapy, second and third line regimens produce lower response rates of shorter duration. At the present time, optimal chemotherapy for most patients aims at maximizing response rates while limiting drug-induced toxicity.

PROGNOSTIC FACTORS

The demonstrated benefit of adjuvant systemic treatment has prompted intense interest in identifying clinical and laboratory factors that can be used to select patients who do or do not need further therapy because of their prognosis. In addition, there is interest to select patients likely to respond to a particular form of adjuvant systemic therapy. The presence and extent of axillary-node metastases is the best-established prognostic factor[32,33]. The following factors are of prognostic importance in specific series of patients: the status of

oestrogen- and progesterone-receptors[36]; the occurrence of overexpression of the epidermal growth factor receptor[37] or the oncogene erbB2 (HER2 or neu)[38]; the extent of cell surface adhesiolysis, determined by measuring cathepsin D[39]; the amount of urinary plasminogen activator[40]; histologic features such as the histologic or nuclear grade[41,42]; cytokinetic parameters and DNA aneuploidy. Other newer, promising prognostic factors, such as heat-shock proteins[43], nm23[44], collagenase type IV[45] and erbB3[46] will require more study. In addition, there is evidence to suggest that the presence of micrometastases in bone marrow detected by monoclonal antibodies[47] and the presence of new blood vessels in the vicinity of the tumour detected by factor VIII immunohistochemical staining[48] or CD34[49] may be useful in assessing prognosis. The studies to date have been too small to evaluate all these prognostic variables simultaneously, leaving clinicians and patients confused on the tests to rely on.

STEROID-RECEPTORS AND PROGNOSIS

The prognostic significance of steroid-receptor status of the primary breast tumour is still under debate. The prognostic value of oestrogen-receptor (ER) content in tumour tissue with regard to recurrence rate is stronger in studies with relatively short follow-up and in studies in which the estimated disease-free intervals (DFI) were calculated on relatively few recurrences[50-59].
Studies with more prolonged follow-up times suggest that the favourable effect of ER-positivity on recurrence rate is gradually decreasing in time[60,61,62]. It has been suggested that the measurement of progesterone-receptor (PgR) content in breast cancer tissue provides information about prognosis with regard to relapse-free survival[63,64]. Other studies, however, do not confirm this correlation[65,66].

DNA CONTENT AND DNA SYNTHESIS; DNA FLOWCYTOMETRY (FCM)

The cell cycle can be divided in three periods: the G0/1 phase when the cell contains 2 haploid sets of chromosomes (2 C DNA content), the G2/M phase with a double amount of DNA (4 C DNA content) and the S-phase when a cell is in the proliferative phase with the DNA content varying between 2 C and 4 C. The historical consensus from available studies is that duration of the interval of DNA synthesis or S-phase in breast cancer cells is approximately 18 hours, the postsynthetic gap G2 near 4-5 hours, the duration of mitosis 1-2 hours, the G0 resting state and G1 presynthetic gap are variable and the total duration of the cell cycle is 1-4 days[29]. The cell cycle time, i.e. the time from one mitosis to the next, predicted by Thymidine Labelling Index (TLI), would suggest a mean doubling time for breast cancer to be approximately 12 days assuming no exit from the proliferative compartment[67]. Since actual measured doubling times of breast carcinomas are at least 10 times longer[67], the losses from the proliferative compartment must be considerable. The most direct measurement of cell proliferation is the mitotic index (MI), which can be measured directly from routine histologic sections. Because the MI of most breast cancers is extremely low, it is very difficult to measure and would require counting more than 50.000 cells.

The introduction of tritiated thymidine, a precursor for DNA synthesis, enables the S-phase cells to be identified with confidence through the use of autoradiography[68]. The TLI of breast cancer cells is approximately 40 times greater than the MI and can be measured with reasonable accuracy by counting 2000 nuclei in an autoradiograph.

The technique of DNA flow cytometry can provide clinicians with two potentially important pieces of information. In the first place it gives a measurement of the percentage of cells with a DNA content corresponding with the S-phase of the cell cycle. In the second place it gives information about the presence and degree of abnormal DNA content in the breast cancer cell population. Tubiana and Silvestrini have demonstrated the close correlation between thymidine labelling index (TLI) and clinical outcome in breast cancer patients[69,70,71]. FCM and TLI appear to provide similar information about the percentage of proliferating cells[72]. Since there is now a technique for obtaining cellular DNA from paraffin-embedded tissue blocks[73], retrospective analyses regarding the prognostic impact of tumour cell cytokinetics have become possible.
The basic principles of FCM are illustrated in figure 1. Nuclei in liquid suspension are labelled with a fluorescent dye which binds to DNA. These fluorochromated nuclei flow through a narrow orifice allowing only a passage of a single cell and are passed with high velocity through a high intensity light beam which is usually provided by a laser or high pressure arc lamp. The nuclei passing the light beam are able to excite the dye and to emit a light signal of higher wave length, which is collected in a photo-multiplier tube. This tube converts the light signal in an electric signal, which can be made visible on an oscilloscope or expressed in units on an arbitrary scale. In the final analysis, the amount of dye bound in or to one single cell is translated into the height of an electric pulse.

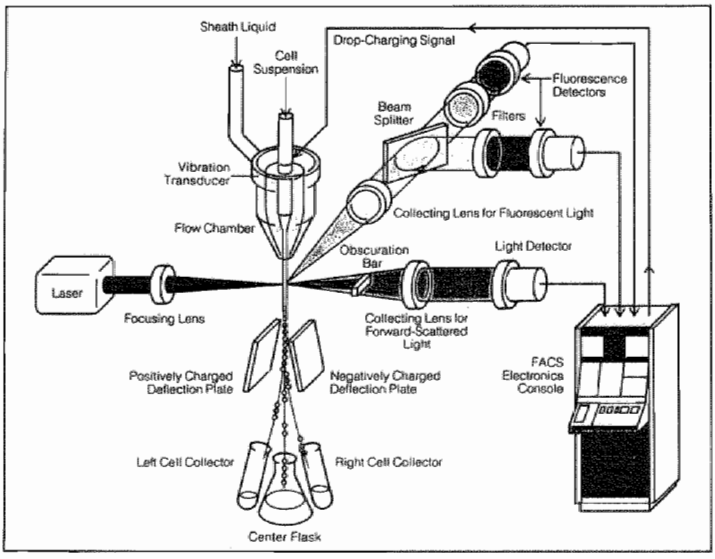

Figure 1 Basic principles of flow cytometry (FCM) by Fluorescent Activated Cell Sorting (FACS).

FCM can be applied to study all those cellular characteristics which can be lured into the binding of a fluorescent dye. The characteristics may be present on the cell surface or in the cytoplasm. FCM can also be applied in staining nuclear material, in particular chromosomal DNA. The DNA histogram reflects a frequency distribution of cells containing a particular amount of DNA. As a consequence, the percentage of cells in the three different phases can be calculated from the DNA histogram.

The measurement of total nuclear DNA content is one of the oldest applications of FCM. For this purpose, nuclei must be stained in suspension with a fluorescent dye that binds specifically and stoichiometrically to DNA, e.g. propidium iodide or DAPI. Cells in tissues must first be disaggregated to obtain a monodisperse suspension that can be measured by FCM. The quality of the measurements depends critically on the quality of the nuclear suspension. Debris particles and unequal uptake of dye in mechanically or chemically damaged nuclei interfere with the resolution of the measurements. Cell preparation procedures for fresh or frozen tissues based on mild enzymatic digestion and treatment with non-ionic detergents such as widely used detergent-trypsin procedure developed by Vindeløv et al. have proved to routinely yield high quality DNA distributions for a wide variety of tissues[74]. This method, however, permits only prospective studies on fresh or frozen tissue samples. This situation has changed with Hedley et al. introducing the pepsin-digestion technique to render archival paraffin-embedded tissue blocks accessible to FCM analysis[72,75,76]. Since the introduction of this technique, a number of clinical studies have been published on the prognostic value of DNA ploidy in a great variety of human tumours. This method has an undeniable role in evaluating the clinical behaviour of tumours with DNA content aberrations. However the usually lower resolution of DNA histograms based on deparaffinized samples may eventually limit the contribution of studies on paraffin specimens[77].

Most groups use the admixed non-neoplastic stromal cells and leucocytes present in most solid tumours as an internal ploidy reference. The position of the DNA diploid cell population in fresh or frozen material can be verified by adding trout or chicken erythrocyte nuclei[78]. This method cannot be applied to deparaffinized samples, due to differences in dye uptake between standard cells and cells from formalin-fixed tumour tissue[79].

The S-phase fraction (SPF) cannot be calculated in a considerable percentage of DNA histograms of tumour samples. Tumours with hypodiploid DNA content as well as low DNA aneuploid ($DNA < 1.15$) and multiploid tumours are not suitable for SPF determination[80,81] and can introduce a bias in the evaluation of correlation between SPF and prognosis[81].

Proliferating non neoplastic cells have in general a lower SPF value than malignant cells causing an underestimation of SPF in DNA diploid or low DNA aneuploid tumours[81,82]. Another pitfall in SPF determination is cell debris arising during the preparation of tumour material for FCM measurements or due to tumour necrosis which causes artificially high SPF values. SPF measurements particularly from paraffin-embedded material should therefore be evaluated with reserve[83] especially if the SPF results will affect the choice of therapy[81]. This is also true if computer programs are used to solve some of the problems mentioned[84,85].

PROBLEMS IN FCM INTERPRETATION

Abnormal DNA content of cells can be identified by FCM when the total abnormal cellular DNA content exceeds 5% of total DNA, depending on the ratio of normal to abnormal cells and the resolution of the DNA histogram. In contrast to karyotyping, balanced chromosomal translocations and small deletions or amplifications cannot be detected by FCM.

The interpretation of DNA histograms is usually not difficult. Caution is required if artifactual G0/G1 peaks are identified, caused by improper preparation. Especially in paraffin-embedded tissues, necrosis can be a potential source of false aneuploid DNA peaks[86]. The resolution of a DNA histogram in discriminating between diploid and aneuploid cells depends on the quality of the individual peaks, which should be narrow and non-skewed. This peak quality is usually expressed as the coefficient of variation (CV) according to the equation:

CV=(100 x FWHM)/(2.35 x peak channel), in which FWHM is the width of the peak at half-maximum. The CV depends primarily on the quality of the nuclear suspensions, the staining condition and the optical system of the flow cytometer. In case of a low CV or a CV>3% with a perfectly symmetrical curve the possibility of a single cell population is high. If the curve is skewed, the possibility of overlapping cell populations is high. Difficulties in interpretation can be overcome by techniques using monoclonals or light scattering properties which permits selective measurement of subgroups[87,88]. Hedley's suggestion to omit measurement if the CV of a DNA stemline in the region if DNA index (DI)=1.00 exceeds 5.5% is followed in most institutes[73,75]. These tumours are classified as a separate entity.

A tetraploid tumour cell population, overlapping the G2M peak of the normal cells is difficult to diagnose. If the tetraploid tumour cell population is accompanied by its own G2M compartment, the problem is easily solved. Beerman et al. suggested to apply a cut off point of 20% for the size of the G2M fraction to discriminate such peaks from a DNA diploid G2M compartment[89].

Hypodiploidy is a clinically important phenomenon since loss of genetic material is recognized as a contributing cause to malignant progression. Thus, cells with less than normal DNA may be of clinical importance probably because they may have a high S-phase fraction (SPF)[90,91].

In fresh or frozen tumour samples the position of the diploid DNA peak can be marked by using ploidy reference cells, a method that can not be applied to the examination of paraffin-embedded materials. In this material the most left peak is generally considered to be the diploid DNA peak. A possible method to identify a hypodiploid fraction in deparaffinized tumour samples is to compare the area under the two G0/1 peaks with the estimated proportion of tumour cells in histological control sections[89]. This approach is only applicable if the two G0/1 peaks are sufficiently separated. The reported incidence of hypodiploidy in human breast carcinomas varies between 3-10%[80,91].

Deviations in cellular DNA content (aneuploidy) were expressed as DNA index (DI). The DI was calculated as the ratio of aneuploid to diploid G1/G0 peak channel; in case of $\geq$2 peaks the left sided was always considered as diploid.

FLOW CYTOMETRY AND CLINICAL VARIABLES

1. *DNA Content in Malignant Breast Lesions*
   The majority of breast cancer specimens show one or more aneuploid stem lines. In the literature the percentage of cases with aneuploidy ranges between 40% and 89%[92-100]. These diverging results can be explained by the use of different interpretations of the DNA ploidy pattern. Another possibility can be misinterpretation due to the analysis of limited numbers of nuclei from the same tumour, obscuring intra-tumour heterogeneity.

2. *DNA Ploidy and SPF*
   SPF determined by flow cytometry has a wide range in human breast cancer with median values between 5 and 15% in the majority of the reported studies. Most studies show a correlation between SPF and DNA ploidy with DNA aneuploid breast tumours having significantly higher SPF than DNA diploid tumours[82,101-104]. However, McDivitt et al.[105] could not confirm this correlation and many clinical studies concerning FCM in breast cancer patients did not mention the possibility of a correlation.

3. *Correlation between Histological Types and FCM Parameters*
   The literature pays moderate attention to ploidy and histological types of breast cancer. Medullary carcinomas are frequently aneuploid whereas tubular, lobular and mucinous carcinomas are mostly diploid.
   A good correlation is described between grading and DNA ploidy[106-110] (page 11).

4. *Correlation between Tumour Size and FCM Parameters*
   No consensus has been reached with respect to correlation between DNA ploidy and T-status. A number of studies have shown a clear correlation of DNA aneuploidy and increasing size of the primary breast tumour[94,95,100,110-113]. Not all authors, however, could confirm this correlation[95,109,114-116].
   Tumour size and SPF are not equivocally related to each other. Some authors did find a correlation[93,100,115] while most authors did not[81,102,108,117].

5. *FCM Parameters and Nodal Status*
   There is disagreement in the literature with respect to correlation between DNA index and nodal status. Some found a correlation between nodal status and ploidy[84,109,118] whereas most authors did not find a correlation[93,95,96,111,117]. Arnerløv et al. described a correlation between ploidy and axillary nodal status if perinodal growth was seen around the tumour containing lymph nodes[107].
   With respect to SPF most investigators did not find a difference in SPF between node-negative and node-positive patients[50,72,102,109,113,117,119]. Only Koike et al.[115] described a correlation between proliferation index and lymph node involvement. The mean proliferation index was significantly higher in patients with more than 4 axillary nodes involved in comparison with no nodal involvement. There is one report of lower SPF values for diploid tumours in node-negative breast cancer patients when compared to SPF values in diploid node-positive tumours[82].

6. *FCM Parameters and Histological Grading*
Well differentiated tumours are mostly diploid whereas poorly differentiated tumours are aneuploid. This significant correlation is widely reported[50,95,102,106-110,113,118]. In histologically low grade tumours lower SPF values are described in comparison with high grade breast carcinomas[81,99,101,108,109,117].

7. *FCM Parameters and Steroid-Receptor Status*
In general, a higher frequency of aneuploidy is described in ER-negative breast cancers[94,95,100,109,114]. However, this correlation was not always significant. Stuart-Harris et al.(1985) described a weak relationship between ER and DFI[120] whereas many authors could not find a correlation at all[94,105,115,116,119,121,122]. Although less attention has been paid to the relationship between PgR and DNA ploidy the results appear similar to those with ER-status[95,109,115,116,122].
Equivocal relationship between SPF values and ER-status has been reported. Significantly higher SPF values in ER-negative tumours have been described[85,95,105,113,115] but other investigators could not find a correlation between SPF values and ER-status[115,119]. The same lack of correlation has been described between SPF values and PgR-status.

FLOW CYTOMETRIC PARAMETERS AND PROGNOSIS OF BREAST CANCER

DNA Ploidy
A complicating factor in comparing studies on the prognostic value of DNA-ploidy consists of heterogeneity of the investigated populations and differences in duration of follow-up. The majority of investigators have reported a positive correlation between DNA diploidy and a longer OS and/or DFS[81,91,93,94,100,101,104,107,110-112,117,121,123,124]. Other investigators, however, have failed to detect a significant relationship between DNA ploidy and DFS or OS[94,115,120,125]. A number of studies in node-negative breast cancer did not show a significant influence of DNA ploidy on survival[94,126,127] whereas other studies did[80,100,106,112,117,128]. The reason for this discrepancy remains unclear[80,112,117,128].

SPF
Most studies have shown a good correlation between SPF and disease-free and/or overall survival[80,81,101-103,115-117,129]. However, SPF does not always appear to be an independent prognostic factor. Sometimes, SPF was lost in multivariate analysis when histological grading was introduced in the analysis[102,117]. In a multivariate analysis done by Clark et al. for node-negative diploid breast tumours, SPF was the only independent significant predictor of DFS and OS. The classical prognostic factors such as ER-status, age and T-status, did not affect the prognosis beyond the SPF effect in this type of breast cancer patients[80]. However, Clark's conclusions were based on very few events in both the low and high SPF groups.
In node-negative DNA aneuploid tumours SPF does not reach that high predictive value as in DNA diploid tumours[80,103].
Sigurdsson et al. reported SPF to have a major prognostic impact in multivariate analysis in diploid as well as aneuploid node-negative populations[129].

In node-positive breast cancer patients Hedley et al. described a shorter DFS in patients with SPF > 10%, but SPF was not an independent prognostic factor[81].

INTRA-TUMOUR HETEROGENEITY

Intra-tumour heterogeneity in breast cancer has been investigated in a limited number of studies. Assessment of heterogeneity can be of importance since it might be the explanation for the discrepancy between studies regarding the prognostic value and limit the clinical applicability of this technique.
Hitchcock et al. have described 33% (8/24) heterogeneity of DNA ploidy when 3 tissue blocks of the same primary breast tumour was analyzed. When DNA ploidy from metastases was analyzed of the remaining 16 homogenous tumours, 6 of 16 showed heterogeneity in the metastases[130]. In two studies it is indicated that undersampling could lead to underestimation of intra-tumour heterogeneity[130-132]. Beerman et al.[132] found stemline heterogeneity in 27/44 (61%) of the primary tumours when the data of the separate samples (mean 4.9) were pooled. It is therefore quite clear, that the chance of finding ploidy abnormalities, apart from being dependent on the presence and relative frequency of abnormal stemlines, is also dependent on the degree of sampling.

DEGREE OF CONCORDANCE OF DNA PLOIDY BETWEEN PRIMARY TUMOURS AND THEIR METASTASES

There is a high percentage of agreement in the literature (74-90%) in DNA ploidy status between primary tumours and their metastases in the literature[96,133,134]. In two recent studies a concordancy in DNA index between the primary tumour and nodal metastases of respectively 56 and 74 percent of the cases is described[130,132]. As expected the degree of concordance is also dependent on the extent of sampling in both the primary tumour and the metastases. The relative stability in DNA index is also supported by FCM studies in which sequential biopsies were taken from the primary tumour. No changes in DNA index during intervals of several weeks to eight months were observed[135,136].
However, discordancy in DNA ploidy value between primary breast cancer and lymph node metastases have been described as well[96,130,131,133]. It is not clear whether such discordancy reflects clonal selection processes towards certain types of ploidy abnormalities.

The SPF values in primary tumours and metastases are equal in approximately 2/3 of the cases and the mean SPF values do not differ significantly[133,134].

THIS THESIS

The work, described in this thesis, sought to contribute to the treatment of patients with primary breast cancer in two ways: by evaluating a combined adjuvant treatment with chemotherapy and a progestagen and by evaluating the prognostic impact of steroid-receptors and DNA FCM derived parameters in a large and homogeneously treated cohort of patients.
In the first part of this thesis, we sought to determine, whether the addition of hormonal treatment with high dose progestagen to adjuvant chemotherapy would improve the prognosis of patients with axillary node-positive primary breast cancer. These patients were part of a large cohort of primary breast cancer patients seen in the South-Eastern Netherlands during a period of 5 years. Premenopausal and postmenopausal women younger than 71 years were treated surgically and selectively with irradiation. Node-positive patients were post-operatively treated with chemotherapy (cyclophosphamide, doxorubicin and 5-fluorouracil) every 4 weeks for 6 months. These patients were randomized into two arms A: no concomitant progestagen and B: concomitant medroxyprogesterone acetate intramuscularly. Endpoints of the clinical study were: disease-free survival (DFS), survival to locoregional relapse, to distant metastases and overall survival (OS)(chapter II and IIa).
This cohort, that is unselected and treated according to this well defined protocol, was also used to investigate the importance of a number of potentially useful prognostic factors. A number of clinical variables were investigated such as: age, oestrogen and progesterone-receptor content, tumour stage (TNM classification) and the number of tumour containing axillary nodes. The prognostic significance of steroid-receptor activity has been studied on 329 node-negative and 320 node-positive patients (see chapter IV). In addition to clinical parameters and steroid-receptor content, special attention was paid to parameters associated with nuclear DNA-content and proliferation. Flow cytometric DNA determination was performed on nuclei isolated from formalin fixed paraffin embedded tumour containing tissue blocks. First, the prognostic significance of DNA-content and S-phase fraction in 329 node-negative and 320 node-positive patients was analyzed and described in chapter IV and chapter V, and subsequently DNA content and the percentage of proliferating cells of primary tumours and their nodal metastases have been examined (chapter VI).

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Chapter II

ADJUVANT CHEMO-HORMONAL THERAPY WITH CYCLOPHOSPHAMIDE, DOXORUBICIN AND 5-FLUOROURACIL (CAF) WITH OR WITHOUT MEDROXYPROGESTERONE ACETATE FOR NODE-POSITIVE BREAST CANCER PATIENTS

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ABSTRACT

Background

The Comprehensive Cancer Center trial 82-01 is a prospective randomized study to investigate the value of the addition of high dose medroxyprogesterone acetate (MPA) to chemotherapy in patients with node-positive operable breast cancer. MPA may be of advantage in this setting because of its activity in oestrogen-receptor (ER)-positive as well as ER-negative tumours and since it may protect against chemotherapy-induced myelosuppression and thus enable maintenance of the appropriate chemotherapeutic scheduling.

Patients and methods

Four hundred eight evaluable patients with node-positive (N+) operable breast cancer (T_{1-3},N_1) were entered in a multicenter randomized trial. Two hundred nine patients were randomized in the MPA- arm and 199 in the MPA+ arm. CAF chemotherapy was given as a short i.v. bolus infusion: cyclophosphamide 500 mg/m^2 i.v. day 1, doxorubicin 40 mg/m^2 i.v. day 1 and 5-fluorouracil 500 mg/m^2 i.v. day 1, q 4 wks x 6. MPA was given intramuscularly (i.m.) 500 mg q d x 28 days, followed by 500 mg i.m. twice weekly during 5 months.

Results

The main side effects of MPA were weight gain with a mean of 5.5 kg as opposed to 1.8 kg in the control group ($p=0.01$) and vaginal bleeding in 30/199 in the MPA+ group and 0 in the MPA- group. MPA ameliorated vomiting grade III, IV (45% vs 28%, $p<0.001$), nausea grade III, IV (50% vs 34%, $p<0.001$) and leucocyte nadir grade III, IV (20% vs 11%, $p=0.003$). Disease-free survival (DFS) after 5 years was 59% in the MPA+ and 49% in the MPA- group ($p=0.12$). Patients ≥ 60 years benefitted most from MPA treatment, in particular if freedom from distant metastases was taken as the endpoint ($p=0.02$). Overall survival (OS) was not significantly different between the two treatment groups ($p=0.18$), but within subgroups analyzed there was an advantage for MPA+ in patients ≥ 55 years ($p=0.002$) and in pT_1 patients ($p=0.045$).

Conclusions

High dose MPA ameliorates CAF side effects and reduces the risk of metastatic disease, especially in elderly breast cancer patients.

INTRODUCTION

In the early 1980s the results of adjuvant chemotherapy studies from the NSABP[1] and the Milan Group[2,3] began to show a statistically significant benefit in terms of treatment failure for women with node-positive breast cancer treated who received chemotherapy as compared to those in the observation group. With the maturation of these studies and the results of subsequently initiated trials it has now become possible to conduct a meta-analysis that has clearly confirmed this benefit[4] for premenopausal and also, although to a lesser extent, for postmenopausal women in terms of both disease-free (DFS) and overall survival (OS). In this time period, data from studies with adjuvant hormonal treatment have only slowly been accumulated. It appears now that adjuvant treatment with tamoxifen improves DFS in node-positive breast cancer patients, but overall survival advantage is more difficult to demonstrate, and in the meta-analysis OS reaches significance only in patients over 50 years of age.

At present, the least understood situation in adjuvant treatment of breast cancer is the use of combinations of cytostatic and hormonal adjuvant therapies. In metastatic breast cancer chemo-hormonal therapy is distinctly superior to chemotherapy alone[5,6]. In the 1980s a number of trials were initiated that sought to investigate the effectiveness of the addition of 1 year of tamoxifen to a CMF-type chemotherapy[7-15]. In general, these studies, although sometimes suggesting a superiority of the combination in subgroups of patients for disease-free interval[16,17] have yielded negative results. Discrepancies in therapeutic findings are still unexplained, but could be related to interactions between chemotherapy and hormonal drugs[18,19] or to the short duration of hormonal therapy[11].

In 1982 we started to address the question of the efficacy of various chemo-hormonal adjuvant therapy regimens because their results were better than those of cyclophosphamide, methotrexate and 5-fluorouracil (CMF)-based combinations[20] in metastatic breast cancer, and because in non-randomized studies, the number of positive lymph nodes and the menopausal status had been observed to have less influence on the efficacy of such adjuvant therapy[21]. We chose a doxorubicin-containing cytotoxic regimen. Medroxyprogesterone acetate (MPA), that had not been adequately tested in an adjuvant setting, was used as hormonal treatment because of its considerable activity in oestrogen-receptor ER-positive and possible activity in ER-negative tumours[6,22]. Because of the bone marrow protective activity of MPA against chemotherapy-induced toxicity, this agent could also make it possible for the appropriate chemotherapeutic dose-intensity to be maintained[23]. Here we report the results of this study after a median follow-up of 42 months.

PATIENTS AND METHODS

Patient Eligibility

Premenopausal, and postmenopausal women younger than 71 years with histologically-proven infiltrative breast cancer treated with a modified radical mastectomy or lumpectomy with postoperative breast irradiation, having one or more

histopathologically-involved ipsilateral axillary lymph nodes, were considered for entry in the protocol. Lumpectomy was performed in patients with a primary tumour of 3 cm or smaller. Postlumpectomy breast irradiation consisted of 50.00 Gy to the total breast with a boost of 14.00 Gy to the operated region. Patients with carcinoma in situ, bilateral breast carcinoma, inflammatory breast cancer or T_4 primary tumour, nodal fixation or supraclavicular nodal involvement and/or evidence of disseminated disease at presentation were ineligible for the study. Other eligibility criteria included a normal bone scan, no previous or concomitant malignancy (except curatively treated (non melanoma) skin cancer or cervix carcinoma in situ), and no pregnancy or lactation at the time of diagnosis of breast carcinoma. Exclusion criteria for entry were haemoglobin level <6.5 mmol/l, leucocyte count $<3.10^9$/l or platelet count $<100.10^9$/l and medical illnesses precluding the treatment options (e.g., recent myocardial infarction, a history of thromboembolism and congestive heart failure). Informed consent was required according to the institutional guidelines. The protocol had been approved by the institutional committees for medical ethics. The patients were staged according to the TNM system adapted by both the Union Internationale Contre le Cancer and the American Joint Commission on Cancer Staging[24].

Randomization
Randomized treatment assignments took place by telephone call to the office of the Comprehensive Cancer Center Limburg. Registration and randomization of patients occurred preoperatively but only patients with postoperative positive nodes were subsequently treated according to the assigned treatment.

Study design
All patients were treated with adjuvant chemotherapy. Randomization determined whether or not patients also received MPA. The adjuvant chemotherapy consisted of six 28-day cycles beginning within 4 weeks after primary surgery. In cases of postoperative breast irradiation, both modalities were combined with chemotherapy starting just prior to the radiotherapy. Each cycle of the CAF regimen consisted of cyclophosphamide 500 mg/m² intravenously (i.v.) on day 1, doxorubicin 40 mg/m² i.v. on day 1 and 5-fluorouracil 500 mg/m² i.v. on day 1. MPA (Farlutal®) was administered daily in a dose of 500 mg intramuscularly (i.m.) days 1 through 28 and twice a week thereafter for a total period of 6 months (figure 1).

Drug dosage modification
Treatment delays of up to 2 weeks were allowed for each cycle to allow for recovery from haematologic toxicity. If on day 28 leucocyte count was $<3.10^9$/l and/or platelets $\leq 100.10^9$/l the CAF chemotherapy course was postponed for 1 week. One hundred percent of the CAF regimen was given if leucocytes were $>3.10^9$/l and platelets $>100.10^9$/l. CAF doses were reduced to 50% if leucocytes were between 2.10^9/l and 3.10^9/l and/or platelets between 50.10^9/l and 100.10^9/l after a one-week postponement. CAF chemotherapy was discontinued if the leucocyte count after a one-week postponement was $<2.10^9$/l and/or platelets $<50.10^9$/l; in such cases the patients went off study. MPA 500 mg intramuscularly daily could be replaced by oral intake of 1.000 mg daily in instances of local pain or local infections. MPA treatment was stopped

when it caused unacceptable toxicity for the patient (e.g., unacceptable weight gain, vaginal bleeding) or thrombo-embolic complications. Toxicity was assessed by the WHO criteria[25].

C : cyclophosphamide 500 mg/m^2 i.v. day 1
A : adriamycin (doxorubicin) 40 mg/m^2 i.v.1 hr infusion day 1
F : 5-fluorouracil 500 mg/m^2 i.v. day 1 q 4 wks
MPA : medroxyprogesterone acetate day 1 through 28
500 mg daily i.m.
twice a week 500 mg i.m. for 5 months

WBC $< 3.10^9$/l and/or platelets $\leq 100.10^9$/l: 1 week postponement

After one week postponement

WBC/Plt	**>100**	**50-100**	**<50**
>3	100%	50%	off prot.
2-3	50%	50%	off prot.
<2	off prot.	off prot.	off prot.

Figure 1 CAF-MPA regimen.

Patient evaluation
Follow-up included a history and physical examination every 3 months during the first 2 years, every 4 months in the third year, every 6 months in the fourth and fifth years and every 12 months thereafter. Blood counts, alkaline phosphatase, gamma-glutamic transferase, lactodehydrogenase, calcium, and erythrocyte sedimentation rate were determined at the same intervals. Chest radiographs and mammography were performed every year.

Patient accrual
Accrual was from May, 1982 through July, 1987. Four hundred eight patients fulfilled the entry criteria and were treated according to the preoperatively assigned treatment arm. Two hundred nine patients have received the non-hormonal treatment arm (MPA-) and 199 patients the MPA+ arm. The small imbalance between the two treatment groups is due to double randomizations, pre- and postoperatively, of 7 patients. Nineteen patients were randomized for MPA+ but never received hormonal treatment. These 19 patients are included in the MPA+ arm. The median follow-up of this report is 42 months.

Statistical methods
The influence of the addition of hormonal therapy on the endpoints of interest: overall survival (OS), disease-free survival (DFS), survival to loco-regional relapse and survival to distant metastases, was primarily analyzed using Kaplan-Meier survival curve estimates and the logrank test[35].
However, as there are some slight imbalances with respect to the distribution of patient characteristics between the MPA+ and MPA- treatment arms, the influence of MPA on

survival was additionally analyzed using Cox's proportional hazards model to control for possible confounding. Essentially the same results were obtained as with the Kaplan-Meier/log rank test analysis. Risk ratios and 95% confidence intervals were estimated using the proportional hazards model to fit only the MPA effect. All Kaplan-Meier curves are presented with 95% simultaneous confidence bands following Hall & Wellner[26]. Confidence bands for two curves, CAF vs CAF+MPA, are adjusted with the Bonferroni inequality to give an overall simultaneous 95% coverage probability. The Cox's proportional hazards model was also used to adjust for confounding while looking for subgroups of patients with good response to CAF+MPA therapy. This subgroup exploration was done through fitting all interaction terms of all relevant variables with MPA using backward elimination with a p-value of 0.10. The subgroups showing a large difference in survival curves are presented. We present here Kaplan-Meier curves estimated for the actual subgroups and not adjusted curves based on the proportional hazards model. Statistics were also calculated for the actual subgroups. We would like to point out that this subgroup search was only made as an inquiry as to possible topics for further research. The assumptions of the proportional hazards regression model are carefully checked using graphical methods and Schoenfeld tests and this results in transforming some prognostic variables to improve fit. Undue influence of individual patients on results was also checked using influence and residual plots[27].

RESULTS

The two treatment regimens are generally well balanced with respect to the analyzed patient characteristics (table I). Checks, controlling for these slight imbalances in treatment comparisons, do not indicate confounding.
The OS curves and DFS curves for all 408 patients at a median follow-up of 42 months are shown in figures 2 and 3 respectively. The DFS and OS were 73% and 80%, respectively, at 42 months.

Table I Patient Characteristics

	Treatment (CAF)			
	MPA-(209)		MPA+(199)	
	No	(%)	No	(%)
Characteristic				
age premenopausal	113	(54%)	123	(62%)
postmenopausal	96	(46%)	76	(38%)
Weight (kg ≥74 kg)	44	(21%)	50	(25%)
ER pos.(≥10 fmol/mg prot.)	148	(71%)	131	(66%)
Tumour stage (AJCC classification)				
I	31	(15%)	26	(13%)
II A + B	144	(69%)	147	(74%)
IIIA	33	(16%)	28	(14%)
T_1 (TNM class.)	54	(26%)	64	(32%)
T_2	136	(65%)	115	(58%)
T_3	19	(9%)	20	(10%)
Number of axillary nodes				
1-4	134	(65%)	135	(68%)
5-9	54	(26%)	46	(23%)
≥10	19	(9%)	18	(9%)
% died from malignancy	56	(27%)	48	(24%)
other causes	21	(1%)	20	(1%)
% relapsed				
locoregional*	23	(11%)	22	(12%)
metastatic	65	(31%)	44	(22%)
locoregional + metastatic	12	(6%)	10	(5%)
chemotherapy dose reduction (0%-10%)	25	(12%)	14	(7%)

* chest wall relapse after mastectomy or mammary relapse after lumpectomy and/or ipsilateral axillary lymph node metastases or ipsilateral supraclavicular lymph node metastases

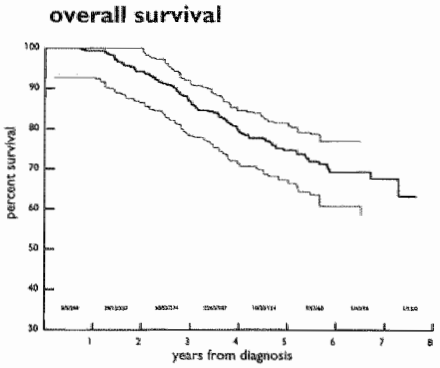

Figure 2 Overall survival (OS) curve for all node-positive patients (n=408).
Dotted lines represent 95% simultaneous confidence bounds.
a/b/c: a. represents the number of patients dying from breast cancer in the one year interval.
b. represents the number of patients without further follow-up or dying from other causes.
c. represents the number of patients alive and still at risk of dying at the end of the one year interval.

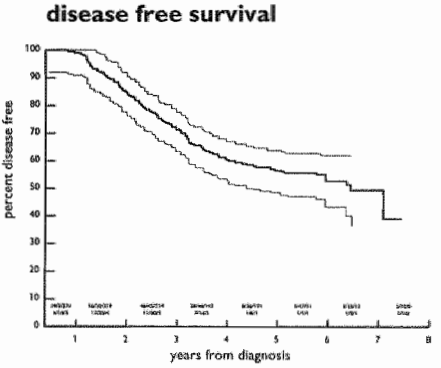

Figure 3 Disease-free survival (DFS) curve for all node-positive patients (n=408).
Dotted lines represent 95% simultaneous confidence bounds.
a/b/c/: a. represents the number of patients with distant metastases or loco-regional recurrence.
b. represents the number of patients without further follow-up or dying from other causes.
c. represents the number of patients still alive and at risk of recurrence at the end of the one year interval.
d/e/f/: d. represents the number of patients with loco-regional recurrence.
e. represents the number of patients with distant metastases.
f. represents d + e
d + e + f = a.

DFS comparisons between the two regimens

A difference in DFS between the two regimens as far as all recurrences are concerned has been found, 59% vs 49% at 5 years in favour of the MPA+ arm, but the difference is currently not statistically significant (p=0.12) (figure 4). An estimate for the relative (MPA+ versus MPA-) rate of occurrence of distant metastases or loco-regional relapses is 0.79. A 95% confidence interval for the true rate is (0.59, 1.07). However, if the comparison is carried out with the occurrence of all relapses for the subgroup of patients aged ≥60 and ≤70 years the two curves differ almost significantly, 62% vs 41% at 5 years in favour of the MPA+ treatment arm (figure 5) (p=0.06). An estimate for the relative (MPA+ versus MPA-) rate of occurrence of all relapses is 0.57. A 95% confidence for the true value is (0.32, 1.03). This difference is even more marked for the occurrence of distant metastases as endpoint in patients aged ≥60 and ≤70 years (p=0.02). The curves diverge from the first year of follow-up and a slightly more than 10% difference can be seen after a follow-up period of 5 years.

No significant differences in disease-free survival can be found in this study within subgroups according to tumour size, steroid-receptor value (the relative rate of recurrence is 1.02 for ER and 0.83 for PgR) or number of positive nodes.

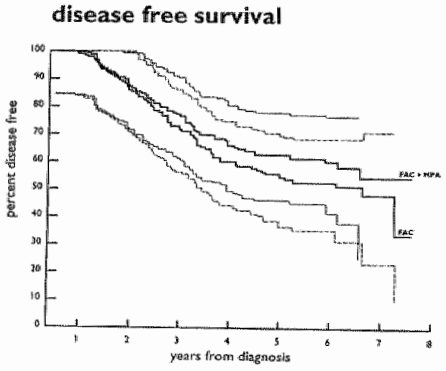

Figure 4 Disease free survival (DFS) curves for both treatment arms for all types of relapses (locoregional, distant metastases and both types simultaneously) (p=0.12). Dotted lines represent 95% simultaneous confidence bounds.

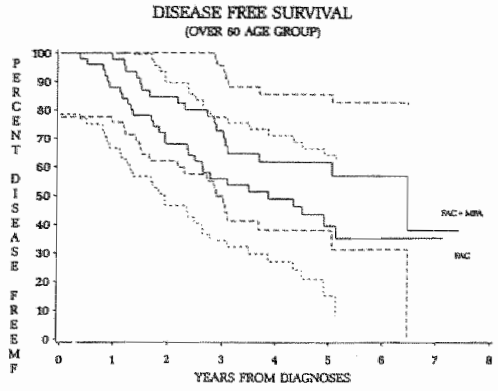

Figure 5 Disease free survival (DFS) curves for both treatment arms for all types of relapses in patients ≥60 and ≤70 years. Difference in favour of the FAC + MPA treatment arm (p=0.06).
Dotted lines represent 95% simultaneous confidence bounds.

Overall survival (os) comparisons between the two regimens
There is currently no significant OS benefit for either of the two regimens (figure 6). An estimate of the relative rates (MPA+ versus MPA-) of cancer related death is 0.88. A 95% confidence interval for the true rate is (0.60, 1.30). There are no differences within subgroups of stage T_2 and T_3, ER or PgR status or the number of positive nodes. However, within the subgroups analyzed there is a survival advantage for CAF + MPA over CAF in elderly patients, statistically most significant in the group of patients ≥55 and ≤70 years of age (p=0.002) and in pathologically staged T_1 patients (p=0.045).

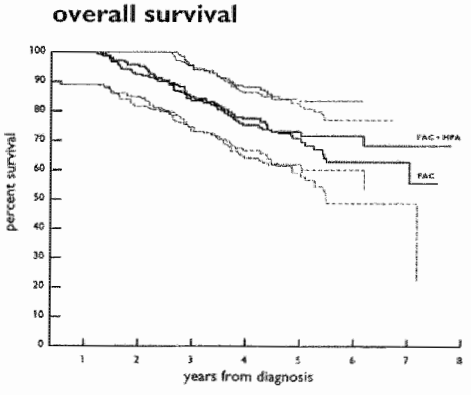

Figure 6 Overall survival (OS) curves for both treatment arms. Only patients who died because of progressive breast cancer were analyzed. No difference between the two survival curves.
Dotted lines represent 95% simultaneous confidence bounds.

Toxicity of the treatment

Six cycles of chemotherapy were administered to all patients. In the MPA+ treatment group 18 (9%) patients discontinued MPA treatment during the adjuvant course, 2 patients in the first month, 12 in the third, 3 in the fourth and 1 in the fifth month. MPA-related toxicity has been assessed by comparing CAF + MPA with CAF. The major side effects encountered are shown in table II. The addition of MPA is associated with significantly less nausea ($p<0.001$), vomiting ($p<0.001$) and grade III and IV nadir leucocytopenia ($p=0.003$). Four weeks after initiation of chemotherapy no significant differences in leucocyte count could be found. No differences were observed in platelet count and granulocytopenia grade III and IV toxicity. In the CAF + MPA treatment group a mean weight gain is recorded of 5.5 kg whereas weight gain in the CAF treatment group did not exceed 1.8 kg (table 2). The CAF chemotherapy was well tolerated with 97% of the planned dose given for patients without MPA, and 98% to those with MPA. Only 5%, 10 patients, (without MPA) and 2%, 4 patients (with MPA) discontinued CAF chemotherapy prematurely.

Table II Major Side Effects Occurring During Treatment with each Regimen (%)

Side Effect	CAF 209 No		CAF + MPA 199 No		p-value
Infiltrate in injected muscle			11	(6%)	
Vomiting (gr.III, IV)	97	(46%)	57	(28%)	<0.001
Nausea (gr.III, IV)	106	(51%)	68	(34%)	<0.001
Leucocyte nadir (III, IV)	42	(20%)	22	(11%)	=0.003
Leucocyte count at 4 wks after treatment (II, III)	23	(11%)	16	(8%)	N.S.
Granulocytes nadir (III, IV)	38	(18%)	40	(20%)	N.S.
Granulocytes at 4 wks (II, III)	25	(12%)	18	(9%)	N.S.
Platelets nadir (I, II)	2	(11%)	2	(1%)	
Platelets at 4 wks (I)	2	(1%)	2	(1%)	
Vaginal bleeding	0	(0%)	30	(15%)	
Weight gain (kg)	1.8		5.5		=0.01

DISCUSSION

Although no survival or disease-free survival difference for all treated patients is observed, the current trial has demonstrated a therapeutic benefit of MPA added to CAF in terms of OS in elderly (≥55 years) patients. Only one other trial has studied a similar question[28]. In that study, MPA added efficacy to CMF treatment; this effect is restricted to patients over 50 years of age. The Early Breast Cancer Trialists' Collaborative Group has reported on the combination of tamoxifen with chemotherapy, mostly of the CMF type[4]. Whereas results of earlier studies of this type using 1 year of tamoxifen treatment were generally negative[7-15], the meta-analysis of trials with an average of 2 years of treatment shows a reduction in odds of recurrence of 7% for patients under and 28% for patients over 50 years of age. Therefore, as with MPA combined with CAF, tamoxifen

appears to add efficacy to the CMF type of adjuvant chemotherapy in elderly patients with breast cancer. Direct comparisons between the efficacy of MPA and tamoxifen in adjuvant treatment are not available but might be worth performing in view of the increase in response rates with MPA over those with tamoxifen in patients with metastatic disease[22]; this, however, has not been uniformly found[29] and may be dose-related[30].

MPA does not appear to significantly affect the occurrence of locoregional relapse. This is also reflected in the finding that the MPA effect is positively associated with the presence of small primary tumours (T_1;$p=0.05$) for survival in this group. The locoregional failure rate of 11%-12% in our study after a median follow-up of 42 months is comparable to that reported by the Italian Breast Cancer Adjuvant Chemo-Hormone Therapy Cooperative Group, Grocta[13] and the North Central Cancer Treatment Group in their trial of combined chemo-hormonal therapy[9]. In both these studies the addition of tamoxifen for 5 years (Grocta) or 1 (NCCTG) year did not improve locoregional control (10% relapse rate for Grocta and 13% for NCCTG) over that of chemotherapy alone. We conclude that high-dose MPA offers no advantage over tamoxifen with respect to locoregional control in patients treated with adjuvant chemotherapy.

The meta-analysis has provided evidence that the adjuvant effect of tamoxifen is dependent (though not completely) on the presence of oestrogen-receptors[4]. This may in part explain why in our study elderly patients responded to MPA adjuvant treatment, since this group tend to have higher ER-levels. Oestrogen-receptor content, however, did not predict for MPA efficacy. In the current study MPA was given over a period of time which, in light of current knowledge, was probably too short for provision by an endocrine mechanism of a meaningful adjuvant effect.

P-glycoprotein mediated multidrug resistance (MDR) may be important in breast cancer patients, particularly during treatment with anthracyclines[31]. There is evidence that tamoxifen can reverse MDR[32]. Until now there have been no clinical studies of the influence on MDR by high doses of MPA, but in vitro studies suggest that progesterone interacts with P-glycoprotein and is capable of reversing drug resistance in MDR-positive cells[33]. Results of the current study using short-term high-dose MPA concurrently with an anthracycline-containing adjuvant chemotherapy may be an expression of this interaction at the clinical level.

The addition of MPA to CAF is associated with a higher incidence of cushingoid changes, weight gain and local infections due to the intramuscular application of MPA. These side effects caused 9% of treated patients to discontinue treatment. A beneficial effect of MPA in this trial is bone marrow protection, in particular an amelioration of the white blood cell nadir. However, this did not lead to a higher dose-intensity and therefore is not the explanation for the differences in efficacy. Nausea and vomiting were partly prevented by the addition of MPA. This is probably due to the corticosteroid activity of the drug[34].
In conclusion, this trial suggests a beneficial effect of MPA in combination with chemotherapy on the occurrence of distant metastases in stage II breast cancer patients.

Currently the trial does not show an overall survival advantage but the present trend in favour of the MPA plus CAF treatment group may well become a significant difference after a longer follow-up period. As high-dose MPA ameliorates CAF side effects and improves the efficacy when combined with CAF chemotherapy in the elderly breast cancer patients, the combination of MPA plus chemotherapy deserves further investigation in elderly breast cancer patients, irrespective of steroid-receptor status.

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Chapter IIa

ADJUVANT CHEMO-HORMONAL THERAPY WITH CYCLOPHOSPHAMIDE, DOXORUBICIN AND 5-FLUOROURACIL (CAF) WITH OR WITHOUT MEDROXYPROGESTERONE ACETATE FOR NODE-POSITIVE CANCER PATIENTS.UPDATE AT 7 YEARS FOLLOW-UP

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ADJUVANT CHEMO-HORMONAL THERAPY WITH CYCLOPHOSPHAMIDE, DOXORUBICIN AND 5-FLUOROURACIL (CAF) WITH OR WITHOUT MEDROXYPROGESTERONE ACETATE FOR NODE-POSITIVE CANCER PATIENTS. UPDATE AT 7 YEARS FOLLOW-UP

LETTER TO THE EDITOR

The Comprehensive Cancer Center Limburg trial 82-01 is a prospective randomized study to investigate the value of the addition of high-dose medroxyprogesterone acetate (MPA) to CAF chemotherapy in patients with node-positive (N+) operable breast cancer (T_{1-3}, N_1). Results of 408 evaluable patients, after a median follow-up of 42 months, have been published in Annals of Oncology[1] and can be summarized as follows: high dose MPA ameliorates CAF side effects and reduces the risk of metastatic disease in elderly breast cancer patients. Patients ≥ 60 years benefitted most from MPA treatment in particular if freedom from distant metastases was taken as endpoint ($p=0.02$). Overall survival (OS) showed a significant advantage in patients ≥ 55 years ($p=0.002$). In this letter we report the updated results after a follow-up of 7 years.

After a median follow-up of 84 months the conclusions of the study remain unchanged. No differences in disease-free survival (DFS), distant metastases-free survival and OS were found for the whole group of patients (p-values were respectively 0.12, 0.12 and 0.18). OS-curves of all patients either or not treated with MPA are shown in figure 1.

Subset analysis revealed a significantly better DFS for the patient group aged between 40 and 60 years compared to the group ≤ 40 or >60 years ($p=0.002$). This difference is MPA-treatment independent.

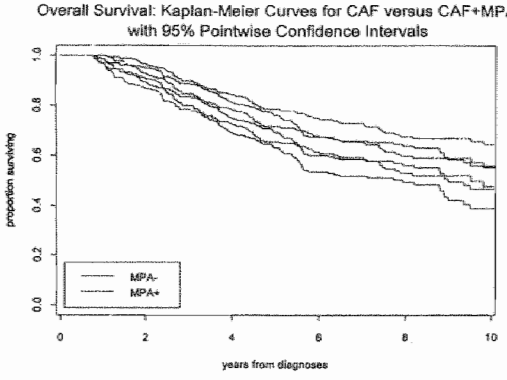

Figure 1 Overall Survival (OS) curve for all node-positive patients in both treatment arms. No statistically significant differences between both treatment arms ($p=0.12$). For both survival curves 95% confidence bounds are drawn.

Patients ≥60 years showed a significantly longer DFS and OS when MPA is added to CAF chemotherapy (p-values respectively 0.05 and 0.008) (figure 2).
In contrast, in the subgroup of patients <40 years, addition of MPA to chemotherapy proved to be detrimental, the relative risk (RR) for relapse of breast cancer was 1.6 versus 1.1 for patients with or without MPA respectively, while the RR in the group ≥60 years was lower (0.7 versus 1.0) in favour of the MPA-treated group.
In conclusion, this trial suggests a beneficial effect of MPA in combination with chemotherapy in elderly patients (≥60 years). This beneficial effect may in part be explained by higher oestrogen-receptor (ER) levels in elderly breast cancer patients. In young breast cancer patients (≤40 years) MPA added to adjuvant chemotherapy has a detrimental effect, possibly caused by the protective effect of MPA on ovarian function during CAF chemotherapy[2], preventing CAF chemotherapy to cause chemotherapy induced ovarian ablation. An alternative explanation may be that MPA reduces the cellular ER- and PgR-content in breast cancer cell lines[3]. This down regulation of ER content in pre-menopausal breast cancer patients could have a negative influence of endogenous oestrogen on tumour-cell cycle (lower percentage of tumour cells in the proliferative phase) causing less effect of adjuvant chemotherapy on tumour cells in premenopausal patients.
The previously described bone marrow protective effect of MPA[1] is recently supported by two studies demonstrating in vitro that MPA exerts a cell cycle arrest of haematopoietic precursors protecting them from the toxicity of chemotherapy[4] and in vivo that MPA induces a mitotic arrest in haematopoietic stem cells[5].
The combination of MPA and chemotherapy deserves further exploration in postmenopausal breast cancer patients.

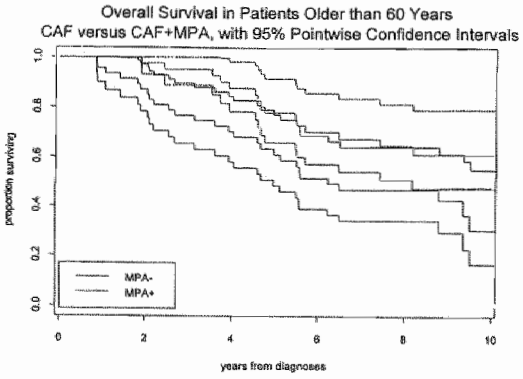

Figure 2 Overall survival (OS) curve for both treatment arms in patients ≥60 and ≤70 years. Differences in favour of the CAF + MPA treatment arm (p=0.008). For both survival curves 95% simultaneous confidence bounds are drawn.

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Chapter III

THE PROGNOSTIC SIGNIFICANCE OF STEROID- RECEPTOR ACTIVITY IN TUMOUR TISSUES OF PATIENTS WITH PRIMARY BREAST CANCER

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THE PROGNOSTIC SIGNIFICANCE OF STEROID-RECEPTOR ACTIVITY IN TUMOUR TISSUES OF PATIENTS WITH PRIMARY BREAST CANCER

ABSTRACT

Introduction
The prognostic significance of steroid-receptor activity is still under debate. Discrepancies in results are probably due to the small numbers of patients, heterogenous patient populations and short follow-up.

Aim
In this study we investigated the prognostic significance of oestrogen- and progesterone-receptor (ER and PgR) as a continuous variable in a homogeneous patient population.

Results
The prognostic significance of steroid-receptor activity was studied on 329 node-negative and 320 node-positive unselected breast cancer patients. In node-negative patients, ER-values of primary tumours between 100 and 400 fmol/mg protein appeared to be a significant predictor for low risk of recurrence, whereas high ER (>400) revealed an unfavourable prognosis. However, the classical cut-off level of ER (<10 fmol/mg) had no prognostic significance. In patients receiving adjuvant chemotherapy, being the node-positive breast cancer patients, the classic cut-off value of ER (10 fmol/mg protein) predicts significantly for DMFS (distant metastases free survival) and OS (overall survival) only in the first four years of follow-up after diagnosis. PgR is a time dependent prognosticator in node-negative breast cancer patients (cut-off point for PgR 80 fmol/mg). In node-positive breast cancer patients treated with chemotherapy or a combination of chemo- and hormonal-therapy, PgR values lower than 60 fmol/mg had a worse prognosis.

Conclusion
The results show the poor performance of standard cut-off points for ER and PgR positivity in predicting prognosis. Better prognosis is related to higher receptor levels but this relation is predominantly time dependent. Moreover, patients with very high ER levels have a prognosis that is worse as compared to intermediate ER levels. This finding might be due to chance and requires independent confirmation. Standard cut-off points for steroid-receptors should be used cautiously to select patients for prognosis.

INTRODUCTION

The prognostic significance of measurement of steroid-receptor content i.c. oestrogen-receptor (ER) and progesterone-receptor (PgR) content in breast cancer with regard to recurrence rate is still under debate. Most studies indicate a more favourable prognosis for patients with ER-positive tumours[1-26]. However, in most of these studies the estimated disease-free intervals (DFI) were calculated on few recurrences, which makes statistical analysis less reliable. Studies with more prolonged follow-up suggest that the favourable effect of ER-positivity on recurrence rate decreases gradually in time[27-31]. One study[32] reported on a gradual but sustained risk of relapse during the full length of the follow-up in ER-positive patients, whereas ER-negative patients developed metastases preferentially in the first three years of follow-up. ER-content was not found to be an independent prognostic factor in two multivariate analyses[33,34]. However, in a large scale prospective study (n=645) ER and PgR were found to have an independent prognostic value in postmenopausal patients. In premenopausal patients PgR but not ER appeared to have independent prognostic value[27].

Node-negative breast cancer patients, which lack ER's appeared to be at a disadvantage in terms of both disease-free survival (DFS) and overall survival (OS)[10]. A multivariate analysis of over 1600 stage I breast cancer patients demonstrated that ER-status is the most significant independent predictor of DFS and OS[21]. However, in a large prospective study of 1157 node-negative patients ER status had only modest prognostic significance[24]. The latter studies[21,24] included breast cancer patients from various institutions who were treated with different schedules of chemotherapy and surgical procedures.

In the present study the results are shown from an unselected breast cancer patient population in which the diagnostic procedure, locoregional treatment and eventually systemic adjuvant treatment were standardized according to the rules of the protocol. The median follow-up time was 84 and 96 months for node-positive and node-negative patients respectively. Steroid-receptor determination was performed in one single institute and its prognostic value was studied as a continuous variable. In this study, all prerequisites for the proper evaluation of the prognostic significance of steroid-receptors are present.

PATIENTS AND METHODS

All patients included in this study were entered in the Comprehensive Cancer Center Limburg Study 82-01 for breast cancer patients from May '82 until July '87. Premenopausal and postmenopausal node-negative women (n=329) younger than 71 years with histologically proven infiltrative breast cancer were treated with a modified radical mastectomy or excisional biopsy and selective breast irradiation. From these node-negative patients 91 (27.6%) underwent a conservative breast surgery including axillary node dissection, 235 (71.4%) underwent a modified radical mastectomy with axillary node dissection and 2 (0.6%) a radical mastectomy.

In the same study 320 axillary node-positive breast cancer patients (pre- and postmenopausal but younger than 71 years) were randomized between chemotherapy alone and chemo-endocrine therapy postoperatively[46].

Surgical treatment consisted either of conservative breast surgery with axillary node dissection (n=45), modified radical mastectomy with axillary node dissection (n=268) or radical mastectomy (n=2). From 5 patients the surgical procedure could not be detected from the hospital records. All patients who underwent conservative breast surgery had complete radiation of the breast.

Postoperative (adjuvant) treatment was randomly allocated into two arms:

A. Chemotherapy consisting of cyclophosphamide 500 mg/m^2, doxorubicin 40 mg/m^2 and 5-fluorouracil 500 mg/m^2 on day 1 every 4 weeks.
B. The same chemotherapy in combination with medroxyprogesterone acetate (MPA) daily 500 mg i.m. (intramuscularly) for 28 days followed by 500 mg i.m. twice a week for 5 months.

In both arms 6 cycles of chemotherapy were given.

The ER and PgR assays were all performed on histologically proven breast cancer tissues using the dextran-coated charcoal method with multiple-point scatchard-plot analysis[38]. For all the assays the minimum cytosol protein concentration was 2 mg/ml cytosol. Determination of ER and PgR content was possible in 303/329 and 255/329 node-negative patients respectively.

In node-positive breast cancer patients ER and PgR assay was possible in 305/320 and 249/320 patients respectively (table I). PgR assays were only used from 1984, which explains the discrepancy in missing numbers of ER and PgR content.

Table I Distribution of parameters in node-negative and node-positive breast cancer patients.

	Node-neg. (n=329)		Node-pos. (n=320)	
Age (years)				
≤50	134	(40.7%)	134	(41.9%)
>50	195	(59.3%)	186	(58.1%)
UICC Stage				
pT_1	189	(57.4%)	91	(28.4%)
pT_{2-3}	140	(42.6%)	229	(71.6%)
ER status (fmol/mg prot)			ER status Node-pos.	
≤100	215	(65.3%)	≤10	119 (37.2%)
100-400	62	(18.8%)	>10	186 (58.1%)
>400	26	(7.95%)		
missing	26	(7.95%)	missing	15 (5.7%)
PgR status (fmol/mg prot)			PgR status Nod-pos.	
≤80	168	(51.1%)	≤60	169 (52.8%)
>80	87	(26.4%)	>60	80 (25.0%)
missing	74	(22.5%)	missing	71 (22.2%)
Ploidy				
diploid	137	(41.6%)	89	(27.8%)
aneupl/multipl	163	(49.5%)	203	(63.4%)
missing	29	(8.9%)	28	(8.8%)
S-phase fraction				
≤8%	99	(30.1%)	88	(27.5%)
>8%	120	(36.5%)	70	(21.9%)
missing	110	(33.4%)	162	(50.6%)

Apart from the clinical parameters that may predict the prognosis also data derived from DNA flow cytometry (FCM) were included in the analysis. FCM determination of DNA levels and S-phase fraction was performed in nuclei isolated from paraffin embedded tissues[50,51].

STATISTICAL ANALYSIS

To investigate the prognostic significance of ER and PgR and other clinical parameters in this study, Kaplan-Meier estimates of survival curves, log rank test for comparing two or more groups and the Cox proportional hazards regression were used. The analyses were done using the statistical packages SAS (SAS Institute Inc., Cary, NC, USA) and S-plus (Statistical Sciences Europe, Oxford, UK).
Routine univariate analyses were done to compare survival curves for quartile and quintile cut-off points. Secondly all parameters were included in a Cox regression analysis with respect to OS and DMFS (distant metastasis-free survival) using standard cut-off points of 10 fmol/mg protein for ER and PgR. Then the assumptions of the Cox regression model, log-linearity of effect and proportional hazards, were carefully checked using tests based on Schoenfeld residuals and by fitting generalized additive models, plotting survival functions and residuals and by smoothing of partial residuals.
This regression analysis suggested non-linearity and/or time dependence for ER and PgR. In the group of node-negative breast cancer patients non-linearities and time dependence were modelled by taking cut-off points of 100 and 400 for ER to define the patient groups $ER \leq 100$, $100 < ER \leq 400$ and $ER > 400$ and a cutpoint of 80 for PgR to compare patients with $PgR \leq 80$ to patients with $PgR > 80$ for the first 4 to 5 years after diagnoses and the period after 5 years of follow-up.
In the group of node-positive patients non-linearities and time dependence were adequately described by taking a cut-off point of 10 for ER and comparing $ER \leq 10$ with $ER > 10$ and by taking a cut-off point of 60 for PgR and comparing $PgR \leq 60$ with $PgR > 60$.
Here there was a suggestion of time dependence for the effect of ER, showing a difference in survival between the above groups during the first 4 to 5 years of follow-up, the difference disappearing thereafter.
The proportional hazards regression p-values are the end result of data exploration and should not be interpreted as direct test of a hypotheses of no prognostic effect. The Kaplan-Meier curves and logrank test presented are based on the cut-off points used in the regression analysis.

RESULTS

Node-negative breast cancer patients
Proportional hazards regression analysis including ER and PgR as potential prognostic factors and using cut-off points of 10 fmol/mg protein for both did not show any association of ER and PgR with either OS or DMFS in the group of node-negative breast cancer patients.

For the 329 node-negative patients in the study we summarized these relationships by taking cut-off points for ER at 100 and 400 fmol/mg protein and comparing the ER ≤ 100 and the ER > 400 patient groups with the 100 < ER ≤ 400 patient group. The ER ≤ 100 patients and the ER > 400 patients had hazard ratios of 2.9 (95% confidence interval (CI) 1.2-7.1), and 3.7 (95% CI 1.2-11.1), compared with the 100 < ER ≤ 400 group of patients for OS. The hazard ratios were 2.1 (95% CI 0.95-4.65), respectively 2.9 (95% CI 1.0-8.2) for DMFS. A cut-off point of 80 fmol/mg protein for PgR suggested that node-negative patients with PgR ≤ 80 had a higher risk than node-negative patients with PgR > 80 up to about 4 to 6 years after diagnosis but a lower risk after about 5 to 6 years after diagnosis, with respect to both OS and DMFS.
The results of the proportional hazards regression analyses are shown in table II. The likelihood ratio test for zero effect of ER on the regression has p-values of 0.02 (OS) and 0.07 (DMFS) and the equivalent test for PgR with time dependence has p-values of 0.002 (OS) and 0.02 (DMFS). Kaplan-Meier curves using these cut-off points for ER and PgR are shown in figure 1 and 2 and they clearly suggest the above mentioned phenomena.

Table II Multivariate survival analysis (time-dependent) in node-negative breast cancer patients.

		DMFS			OS	
Parameter	RR	95% CI	p=value	RR	95% CI	p-value
PgR > 80 vs ≤ 80 follow-up > 5 yr	3.33	1.25-8.87	0.02	4.13	1.75-9.75	0.0004
ER ≤ 100 vs 100-400	2.10	0.95-4.65	0.06	2.89	1.18-7.07	0.02
ER > 400 vs 100-400	2.92	1.04-8.19	0.04	3.66	1.21-11.06	0.02
Aneupl + multipl vs dipl. follow-up < 4 yr	2.71	1.24-5.90	0.008	2.47	1.24-4.90	0.01
pT_{2-3} vs pT_1 follow-up > 3 yr	2.42	1.21-4.84	0.01	2.24	1.26-3.99	0.006

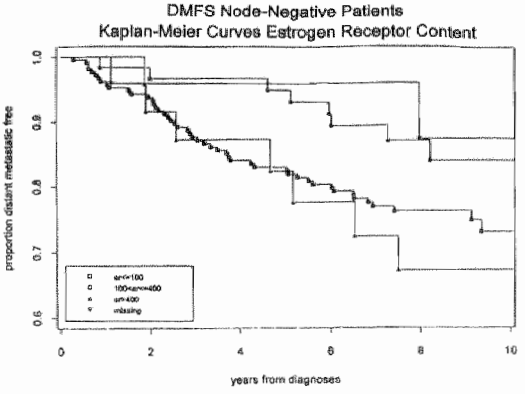

Figure 1 Kaplan-Meier distant metastases-free survival curves in axillary node-negative breast cancer patients.
Cut-off point of oestrogen-receptor (ER) are 100 and 400 fmol/mg protein.

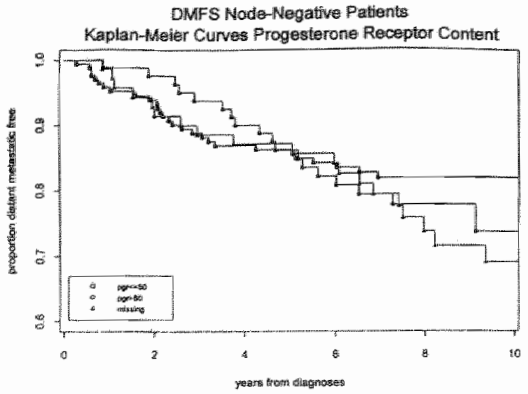

Figure 2 Kaplan-Meier distant metastases-free survival curves in axillary node-negative breast cancer patients.
Cut-off point of progesterone-receptor (PgR) is 80 fmol/mg protein.

Node-positive breast cancer patients

In the node-positive breast cancer patients the group of patients with ($ER \leq 10$) did show a hazard rate ratio significantly larger than 1 compared with patients with ($ER > 10$) for the endpoints OS and DMFS but any cut-off point between 10 and approximately 20 would have shown the same result. Again, we found no association of PgR with either OS or DMFS using a cut-off point of 10.

Data exploration however suggested possible non-linear and/or time dependent effects for both ER and PgR with respect to both OS and DMFS among node-positive breast cancer patients.

For the 320 node-positive patients we summarized the results by taking cut-off points for ER at 10 fmol/mg protein and for PgR at 60. With regard to OS the $ER \leq 10$ patients had about twice the risk of the $ER > 10$ patients, p-value < 0.001, but there was a definite suggestion that the effect of ER was time dependent, the effect being essentially confined to the first 4 to 6 years after diagnosis. $ER \leq 10$ patients had a hazard ratio of 2.9 (95% CI 1.8-4.4) compared with $ER > 10$ patients during the first 5 years of follow-up, the difference essentially disappearing thereafter. With respect to DMFS there was also a suggestion of a time dependent effect of ER, but weaker than with regard to OS. The $ER \leq 10$ patients had a hazard ratio of 1.8 (95% CI 1.1-2.9) compared to the $ER > 10$ patients during the first 4 years of follow-up, the effect again essential disappearing thereafter. With regard to both OS and DMFS the group of patients with $PgR \leq 60$ had about 1.6 to 1.8 times the risk of $PgR > 60$ patients. The respective 95% confidence intervals were 1.0-2.7 and 1.1-3.2. The results of the proportional hazards regression are presented in table III and the Kaplan-Meier curves, figure 3 and 4, illustrate these effects.

Table III Multivariate survival analysis (time-dependent) in node-positive breast cancer patients.

Parameter	DMFS RR	DMFS 95% CI	DMFS p-value	OS RR	OS 95% CI	OS p-value
PgR ≤ 60 vs > 60	1.86	1.07-3.21	0.03	1.64	1.01-2.67	0.05
ER ≤ 10 follow-up < 5 yr	1.78	1.07-2.94	0.03	2.85	1.84-4.43	0.0001
Aneupl + multipl. vs dipl. MPA treated pts	4.45	1.60-12.41	0.004	2.53	1.15-5.56	0.02
SPF > 8 vs ≤ 8% MPA treated pts	0.53	0.19-1.49	0.04	0.37	0.15-0.91	0.03
Age > 50 yr	2.86	1.50-5.45	0.002	1.57	1.08-2.09	0.02
Pos.axill.nodes 4-9 vs ≤ 3	1.91	1.24-2.94	0.003	2.16	1.45-3.23	0.0002
> 9 vs ≤ 9	7.01	3.73-13.16	0.0001	7.47	4.41-12.65	0.0001

OS : overall survival
DMFS : distant metastasis free survival
RR : relative risk
CI : confidence interval
PgR : progesterone-receptor content in fmol/mg protein
ER : oestrogen-receptor content in fmol/mg protein
Aneupl : aneuploid primary breast tumours obtained by DNA flow cytometry
Multipl : multiploid primary breast tumours obtained by DNA flow cytometry
SPF : S-phase fraction
MPA : medroxyprogesterone acetate
Pos.axill.nodes : number of tumour containing axillary lymph nodes

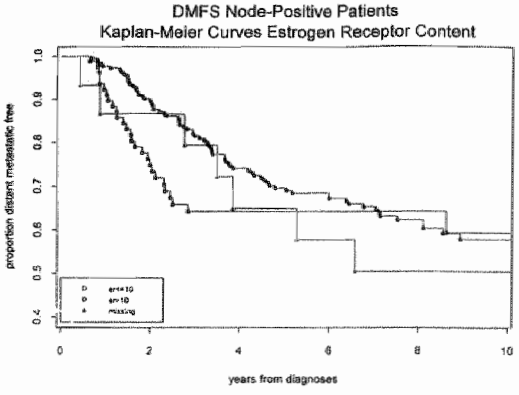

Figure 3 Kaplan-Meier distant metastases-free survival curves in axillary node-positive breast cancer patients. Cut-off point of ER is 10 fmol/mg protein.

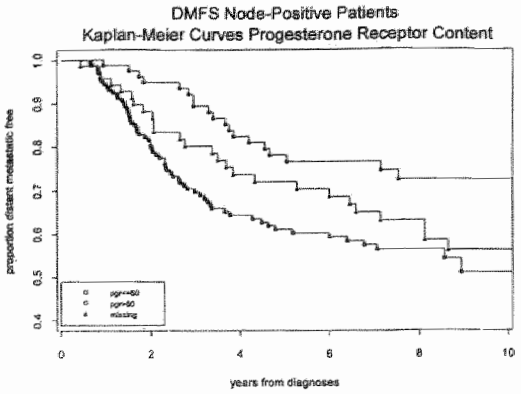

Figure 4 Kaplan-Meier distant metastases-free survival curves in axillary node-positive breast cancer patients. Cut-off point of PgR is 60 fmol/mg protein.

DISCUSSION

The results of the present study suggest an improved DMFS and OS for node-negative breast cancer patients with ER-values between 100 and 400 fmol/mg protein. Patients with ER-values <100 and >400 fmol/mg protein had an inferior survival. These results are time independent.

The conclusion on the highest ER group should be drawn with caution because of the low numbers of patients and events in this group. This finding might be due to chance and requires independent confirmation.

Node-positive breast cancer patients obviously had a poorer prognosis. In this group an ER cut-off point of 10 fmol/mg discriminated between patients with a better and poorer prognosis in the first 5 years after diagnosis. Thereafter, the prognostic impact of the ER virtually disappears.

A review of results in the literature is not conclusive about ER-content as prognosticator in breast cancer patients[1-32]. In most of these studies cut-off points for ER-content of 10 fmol/mg protein are used and many studies had a relatively short follow-up, and often node-negative and node-positive patients are lumped together.

Very few studies addressed the question of other cut-off points for ER in relation to prognosis[15,44,48]. One study[15] showed a significant difference in favour of ER$\geq$200 fmol/mg protein, but the median follow-up period in this study is short (25 months), suggesting the need of a careful interpretation of these results. Thorpe et al.[45] described in postmenopausal patients with ER$\geq$108 fmol/mg had a poor prognosis as well as patients with ER$<$10 fmol/mg, an observation which may be comparable with our results in node-negative breast cancer patients.

A possible explanation of poor prognosis in some patients with high ER-content in the cytosolic fraction can be that in these patients the function of the ER-receptor is impaired. With the biochemical technique the cytosolic ER content is measured. It has been described that in some patients a high cytosolic ER does not reflect a high nuclear ER-content, pointing to an impaired ER-function[47]. Also variants from normal ER have been found with a decreased functional activity[49]. It may be of interest to study ER in the

cytosolic fraction and immunohistochemically simultaneously on the same tumour specimen to reveal the possibility of such discrepancy.

Progesterone-receptor (PgR) content at a cut-off point of 10 fmol/mg, in our study in node-negative breast cancer patients is not a prognostic factor. The time dependent analysis reveals that node-negative patients with PgR contents >80 fmol/mg protein have an increased risk of dying from their disease after a follow-up time of >6 years (RR 4.13). The same phenomenon is seen in this patient group with PgR content >80 fmol/mg protein for DMFS. The relatively low contributions of PgR-content to the final outcome of node-negative breast cancer patients is confirmed by a review of the literature. Small node-negative breast cancer studies reveals that PgR content can not be considered to be a statistical significant prognosticator[31,39-44].
Node-positive breast cancer patients with low PgR levels (<60 fmol/mg protein) showed a high risk for distant metastases and death caused by disease. These patients were postoperatively treated with chemotherapy plus or minus medroxyprogesterone acetate as described elsewhere[46].
In studies including patients receiving adjuvant chemotherapy or chemo- plus hormonal-therapy, PgR predicts the risk of recurrence in a number of these studies[27,39,41-44]. These data indicate that PgR status may be a powerful predictor of the outcome of adjuvant chemotherapy.
In summary this study shows the poor performance of standard ER and PgR cut-off points in predicting clinical outcome. Better prognosis is related to higher steroid-receptor levels but this relation is time dependent. Moreover, patients with very high ER-levels have a worse prognosis compared to intermediate ER-levels.
Standard cut-off points for steroid-receptors should be used cautiously to select patients for prognosis.

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Chapter IV

THE PROGNOSTIC VALUE OF FLOW CYTOMETRY IN WOMEN WITH NODE-NEGATIVE PRIMARY BREAST CANCER

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THE PROGNOSTIC VALUE OF FLOW CYTOMETRY IN WOMEN WITH NODE-NEGATIVE PRIMARY BREAST CANCER

ABSTRACT

There is a need for a prognostic model in node-negative breast cancer patients to individualize post-operative therapy. DNA flow cytometry (FCM) has addressed this problem but results have been variable.
Our results on DNA flow cytometry in a homogeneously treated and diagnosed node-negative breast cancer patient population (n=329), after a median follow-up of 96 months shows ploidy being a time dependent prognostic factor. Patients with aneuploid and multiploid DNA content have a relative risk of 2.9 on distant metastases compared to diploid patients during the first 4 years of follow-up. S-phase fraction was no independent prognostic factor in multivariate analysis.
Apart from ploidy, steroid-receptor status shows a complex but a strong relation with clinical outcome. However, prospective clinical trials in which high risk patients, based on these factors are randomised to either adjuvant treatment or observation, are justifiable.

INTRODUCTION

Approximately 50 to 60% of patients with breast cancer have disease apparently confined to the breast without axillary nodal involvement[1]. The proportion of node-negative breast cancer will probably increase with the implementation of breast screening programs[2]. Although patients with node-negative breast cancer are considered to have a good prognosis, the rate of recurrence in these patients is approximately 30-40% and the 10-year survival rate is around 75%[3].
The use of adjuvant therapy in patients with node-negative breast cancer is still controversial[4-9]. There is a tendency to apply adjuvant therapy in node-negative patients with two or more unfavourable prognostic factors. Unfavourable factors can be defined as tumour size >2 cm[10-14], histologic grade II and III[15], high cell kinetic activity determined by thymidine labelling[16,17] and more recently aneuploidy or high S-phase fraction (SPF) determined by DNA flow cytometry[18-26,27,28].
The goal of this retrospective study is to use DNA flow cytometry in a homogeneously diagnosed group of patients to assess ploidy and S-phase fraction and to evaluate the clinical importance of these measurements in combination with other prognostic factors in order to identify patients with node-negative breast cancer who may have a high or low risk for recurrence.

PATIENTS AND METHODS

Premenopausal and postmenopausal women younger than 71 years with histologically proven infiltrative breast cancer without axillary nodal involvement were included. All patients were treated with a modified radical mastectomy or lumpectomy with postoperative breast irradiation. Lumpectomy was performed in patients with a primary tumour equal to or smaller than 3 cm. Postlumpectomy breast irradiation consisted of 50.00 Gy to the total breast with a boost of 14.00 Gy to the operated region.
Patients with carcinoma in situ, bilateral breast cancer, inflammatory breast cancer or T_4 primary tumour were ineligible. Eligibility criteria included a normal bone scan, no previous or concomitant malignancy (except curatively treated (non-melanoma) skin cancer or cervix carcinoma in situ) and no pregnancy or lactation at the time of diagnosis of breast carcinoma. The patients were staged according to the TNM system adapted by both the Union Internationale Contre le Cancer and the American Joint Commission on Cancer Staging[29].

REGISTRATION

Registration was done by telephone at the office of the Comprehensive Cancer Center Limburg. Registration of patients occurred preoperatively but only patients with postoperative negative nodes were analyzed for this report. All the data were entered into a computerized data base and verified to minimize errors in data entry.

PATIENT ACCRUAL AND TREATMENT

Accrual was from May, 1982 through July, 1987. Threehundred twenty nine patients fulfilled the entry criteria and were treated according to the protocol. Surgical treatment consisted either of conservative breast surgery with axillary node dissection (n=91; 27.7%), modified radical mastectomy with axillary node dissection (n=236; 71.7%) or radical mastectomy (n=2; 0.6%). Postoperative locoregional irradiation was given to patients with central or medial localized breast cancer (parasternal radiation field) (n=121; 36.8%) and in patients with microscopically irradical operation (chest wall irradiation) (n=25; 7.6%). Patients with conservative breast surgery with axillary node dissection were irradiated on the operated breast (n=91; 27.7%). No adjuvant chemotherapy or hormonal therapy was given to these patients. The clinical characteristics are shown in table I. The median follow-up time of the 329 patients is 96 months. As of their last recorded follow-up evaluation, 251 patients were alive (76.3%) and 246 patients were free of distant metastases (74.7%).

PATIENT EVALUATION

Follow-up included a history and physical examination every 3 months during the first 2 years, every 4 months in the third year, every 6 months in the fourth and fifth year and every 12 months thereafter. In the same frequency blood counts, alkaline phosphatase, gamma-glutamic transferase, lacto-dehydrogenase, calcium and erythrocyte sedimentation rate were examined. Chest radiographs and mammography were performed every year.

HISTOLOGICAL EXAMINATION AND LABORATORY ASSAYS

Tumour size was taken from the pathological size after surgery. The histological grade was determined by the method of Bloom and Richardson[30]. Oestrogen- and progesterone-receptor contents were assayed with the dextran-coated charcoal method with Scatchard analysis and calculation according to the method of Lowry[31,32]. A level of at least 10 fmol/mg cytosol protein was considered as receptor positive.
Flow cytometric determination of DNA levels was performed in nuclei isolated from paraffin-embedded tissue[33,34]. Fifty μm sections were cut from formalin-fixed paraffin-embedded tissue blocks of the primary tumours. An adjacent 5 μm section was cut for histological control. DNA content was measured by the method of Vindeløv[35]. Tumours with a single G1 peak were considered to be diploid, whereas evidence of an additional peak indicated aneuploidy. DNA index (DI) was calculated as the ratio of aneuploid to diploid G1/0 peak level. Histograms with coefficients of variation less than 8% were considered of good quality. The proliferative activity (SPF) was calculated by counting the number of cells between the inclination points of the descending G1 peak and the ascending G2/M peak[32,36]. In cases of less than 30% admixture of diploid cells, the percentage of aneuploid S-phase cells was calculated without corrections for the presence of diploid S- and G2/M-phase cells. In case of more than 30% admixture of diploid cells in overlap in diploid and hyperdiploid histograms the percentage of S-phase cells was not calculated.

STATISTICAL ANALYSIS

To investigate the prognostic significance of the clinical parameters included in the study, Kaplan-Meier estimates of survival curves, logrank tests for comparing two or more groups and the Cox proportional-hazards regression model were used.
All analyses were done using the statistical packages SAS (SAS Institute Inc., Cary, NC, USA) and S-PLUS (Statistical Sciences Europe, Oxford, UK).
First, correlations of covariates were studied using Kendall's τau correlations. Routine univariate analyses were done to compare survival curves using quartile and quintile cut-off points for the prognostic variables with respect to the end points 'overall survival' (OS) and 'distant metastatic free survival' (DMFS). Secondly, all prognostic variables were included in a Cox regression analysis with respect to these endpoints. For every variable the assumptions of the Cox regression model, i.e. proportional hazards and loglinearity of effect, were checked.

The proportional hazards assumption was checked through different graphical plots and using tests based on Schoenfeld residuals. Log-linearity of effects was checked through generalized additive modelling and smoothing of partial residual plots.
With regard to both OS and DMFS there was a definite suggestion of non-proportionality for the effect of progesterone-receptor content, ploidy and pT-status and of non-linearity of effect for oestrogen-receptor content. The non-linearity of oestrogen-receptor content suggested taking cut-off points at approximately 100 and 400 fmol/mg protein. The plots also suggested some non-linearity of progesterone-receptor content, S-phase percentage and age: these seemed to be adequately modelled by taking cut-off points at respectively 80 fmol/mg protein, 8% and 50 years of age.
Non-proportionality was dealt with by fitting time-dependent models for progesterone-receptor content, ploidy and pT-status. Differences between pT_2+pT_3 and pT_1 seemed to be 'delayed' for approximately 3 years, the difference between aneuploid/multiploid and diploid seemed to diminish after approximately 5 years and there seems to be a 'switch' in relative risk of progesterone-receptor content >80 (PgR>80) in comparison with (PgR≤ 80) at approximately 4 years for DMFS and approximately 5 years for OS.

RESULTS

Flow cytometry was performed in 300 out of 329 tumour specimens (91.2%). From 29 cases no tumour specimens were available (n=20) or histograms were uninterpretable (n=9). The main characteristics of the missing cases did not differ significantly from those of the patients with interpretable histograms nor was the relapse-free survival or overall survival different for these 29 patients. Overall clinical characteristics are listed in table I.
In 219 of these 300 (73%) patients it was possible to determine the SPF (66.6% of the total number of node-negative breast cancer patients). Of the analyzed patients 137/300 (45.5%) are diploid whereas 149/300 (49.5%) are aneuploid and 14/300 (5%) multiploid.

Table I Clinical and flow cytometric characteristics of 329 patients with node-negative breast cancer

	Number	Percentage
Age (years)		
≤50	134	40.7
>50	195	59.3
UICC stage		
pT_1	189	57.4
pT_{2-3}	140	42.6
ER status (fmol/mg protein)		
≤100	215	65.3
100-400	62	18.8
>400	26	7.95
missing	26	7.95
PR status (fmol/mg protein)		
≤80	168	51.1
>80	87	26.4
missing	74	22.5
Ploidy		
diploid	137	41.6
aneuploid + multiploid	163	49.5
missing	29	8.9
SPF (%)		
≤8	99	30.1
>8	120	36.5
missing	110	33.4

CORRELATIONS OF COVARIATES

Age correlated weakly with oestrogen-receptor content ($r=0.22$, $p=0.003$). A slightly stronger correlation was found between ER and PgR ($r=0.28$, $p=0.001$). SPF correlated with the aneuploid status ($r=-0.26$, $p=0.01$). SPF also showed a weak correlation with age ($r=-0.20$, $p=0.05$) and SPF was also correlated with ER-content ($r=-0.22$, $p=0.03$). There were no significant correlations between all other variables.

UNIVARIATE AND MULTIVARIATE ANALYSIS OF SURVIVAL

The results of univariate analysis are shown in table II. ER-content and pT-status are the most important variables in predicting OS. Statistically significant differences can be found between the different ER-groups ($ER \leq 100$; $100 < ER \leq 400$ and $ER > 400$). There is a significant better overall survival in the intermediate ($100 < ER \leq 400$ fmol/mg protein) oestrogen-receptor group comparing the OS in the ER groups ≤100 and >400. Diploid patients have a better OS in comparison with non-diploid patients ($p=0.02$).

Table II Univariate analysis of prognostic variables in 329 node-negative breast cancer patients: 7 years OS and DMFS

Parameter	No pts.	DMFS No events obs.	DMFS p-value	OS No events obs.	OS p-value
ploidy					
diploid	137	24		18	
aneupl& multipl	163	36		41	
missing	29	5	0.47	7	0.02
Size					
pT_1	189	31		32	
pT_{2-3}	140	34	0.08	34	0.11
ER-status					
≤100	215	48		51	
100<ER≤400	62	8		6	
>400	26	7		7	
missing	26	2	0.08	2	0.04
PgR status					
≤80	168	28		28	
>80	87	18		19	
missing	74	19	0.44	19	0.47
Age					
≤50	134	30		33	
>50	195	35	0.25	33	0.09
S-phase					
≤8	99	19		16	
>8	120	26		26	
missing	110	20	0.87	24	0.47

DMFS analysis shows the same trends for ER-content but the differences are not statistically significant at the 5% level. There is a suggestion (p=0.08) that the group of patients with an extreme high ER-content (>400 fmol/mg protein) have a higher relapse rate compared to patients from the intermediate group.

Kaplan-Meier curves on overall survival and distant metastasis-free survival for ploidy and SPF are shown in figure 1a, 1b, 2a, and 2b.

Multivariate analysis using Cox's proportional Hazards model is done to assess the effects of ploidy, SPF, pT stage, age, and steroid-receptor status on time to relapse and survival. Results are shown in table III. Ploidy status is a time dependent prognostic factor for DMFS; during the first five years of follow-up aneuploid and multiploid breast cancer patients had a relative risk (RR) of 2.7 on distant metastases (p=0.02) (95% confidence interval (CI) 1.24-5.90) compared to diploid breast cancer patients.

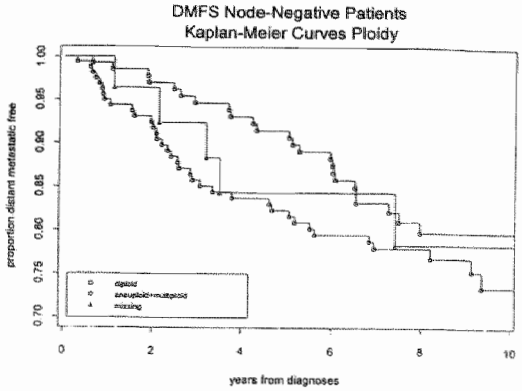

Figure 1a Distant metastases free survival (years) curve for diploid (n=137) and non-diploid (n=163) node-negative breast cancer patients (p-value 0.09).

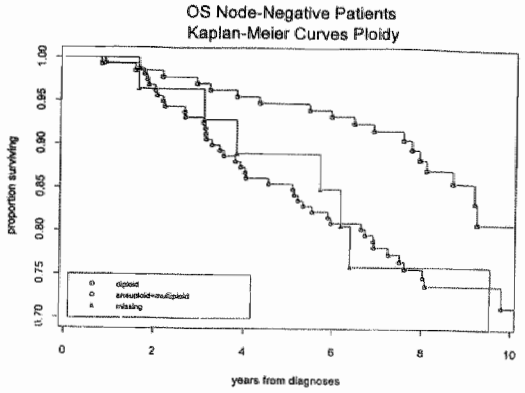

Figure 1b Overall survival (years) curve for diploid and non-diploid node-negative breast cancer patients (p-value 0.02).

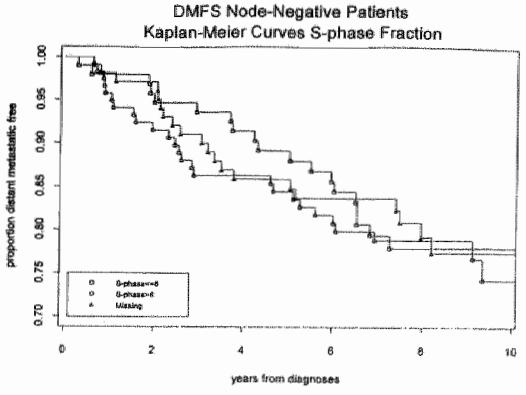

Figure 2a Distant metastases free survival (years) curve for S-phase (SPF) groups ≤8% (n=99) and SPF>8% (n=120) (p-value 0.87).

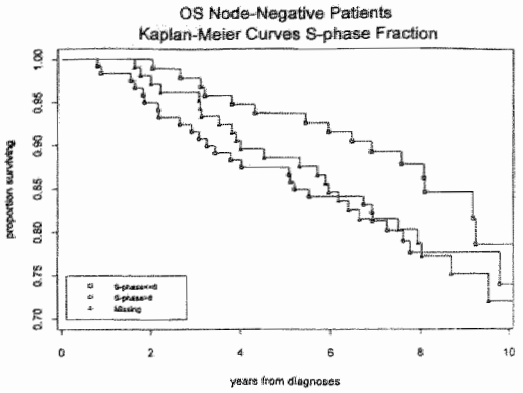

Figure 2b Overall survival (years) cure for SPF groups ≤8% and >8% (p-value 0.47).

Concerning overall survival (OS) ploidy status revealed to be a time independent prognosticator, meaning aneuploid and multiploid patients have a statistically significant shorter OS time compared to diploid patients (RR 2.5, p=0.01; 95% CI 1.24-4.90). SPF is no independent prognostic factor in multivariate analysis, neither for DMFS nor OS in this node-negative breast cancer study. ER-content ≤100 fmol/mg protein and ER-content >400 fmol/mg protein are important prognosticators for OS and in a lesser extent for DMFS. There is a suggestion that patients with PgR≤80 fmol/mg protein have a higher risk for distant metastases during the first 4 years of follow-up, compared to the PgR>80 group, while this risk after 4 years of follow-up is significantly higher for patients with PgR>80 (OS: RR 4.13; 95% CI 1.75-9.75 and distant metastases RR 3.3; 95% CI 1.25-8.87).

pT_2 and pT_3 primary tumours had an impaired survival compared to pT_1 tumours, but the DMFS differences were marginally statistically significant (p=0.06, RR 2.2; 95% CI 1.26-3.99).

The age of node-negative breast cancer patients did not appear to be an independent prognosticator. A combination of variables for unfavourable prognosis are pT_{2+3} + aneuploidy and multiploidy, pT_{2+3} + PgR≤80, ER≤100 and pT_{2+3} + ER>400. Together these groups contain 110/329 (33%) of patients. Their 5-year survival rate is 78% as compared to 92% for the other 219 patients not having these unfavourable characteristics.

Table III Multivariate analysis (Cox proportional Hazards regression) of prognostic covariates of survival in 329 node-negative breast cancer patients followed-up for a median of 96 months

	OS					DMFS			
	ß	p-value	RR	95% CI		ß	p-value	RR	95% CI
pT_{2-3}/>3yr	0.81	0.006	2.24	1.26-3.99	pT_{2-3}/>3yr	0.88	0.01	2.42	1.21-4.84
An+multi	0.90	0.01	2.47	1.24-4.90	An+multi/<5yr	1.00	0.01	2.71	1.24-5.90
ER≤100	1.06	0.02	2.89	1.18-7.07	ER≤100	0.74	0.07	2.10	0.94-4.65
ER>400	1.30	0.02	3.66	1.21-11.06	ER>400	1.07	0.04	2.92	1.04-8.19
PgR>80/<5yr	-0.54	0.29	0.58	0.22-1.57	PgR>80/<4yr	-0.21	0.62	0.81	0.35-1.86
PgR>80/>5yr	1.42	0.001	4.13	1.75-9.75	PgR>80/>4yr	1.20	0.02	3.33	1.25-8.87
SPF≤8	0.13	0.73	1.13	0.56-2.30	SPF≤8	0.20	0.55	1.22	0.65-2.30
Age<50	0.29	0.29	1.34	0.78-2.31	Age<50	0.22	0.43	1.24	0.72-2.14

pT_{2-3}/>3 yr : pathological stage T_2 and T_3, more than 3 years after diagnosis.
An+multi/<5 yr : aneuploid and multiploid tumours, less than 5 years after diagnosis.
Pl.missing/<5 yr : ploidy missing, less than 5 years after diagnosis.
PgR>80/<5yr : progesterone-receptor >80 fmol/mg protein, less than 5 years from diagnosis.
ER≤100 : oestrogen-receptor ≤100 fmol/mg protein.
SPF : S-phase fraction.
ß : parameter estimate.
RR : risk ratio.
CI : confidence interval.

DISCUSSION

In our series of 329 node-negative breast cancer patients aneuploidy but not S-phase fraction was an independent prognostic factor for overall survival after 96 months. Other independent prognosticators were ER≤100 fmol/mg protein, ER>400 and PgR≤80 (early years of follow-up). These factors in combination with pT also predicted a higher chance to develop distant metastases; the highest relative risk (RR 2.7) was observed in patients with aneuploidy. A combination of three factors (ER≤100, ER>400, aneuploidy and pT_{2-3}) identified a cohort with a risk to develop recurrent disease, but the power of these observations is not sufficient to individualize therapy in breast cancer patients. A prognostic model, therefore, established in the way previously described using DNA flow cytometry for node-negative breast cancer patients[37] should be used with caution.

In the past, a number of studies have addressed the question, whether data on ploidy and proliferation obtained with DNA FCM contribute to the prognosis of patients with axillary node-negative primary breast cancer[20-28,38-49]. Results have been variable but in univariate analyses indications for a poorer prognosis for patients with aneuploid tumours were obtained in 15 of 19 studies. Our results confirm this relationship. Patients with aneuploid tumours have an almost four times higher chance to develop distant metastases at 3.5 years (5% versus 19%, p=0.02). Similar differences were obtained in two of the largest other studies dealing with node-negative patients. Sigurdsson et al.[37] found an almost twofold difference (12% versus 23%, p=0.03) in DFS at 4 years in 305 patients,

whereas in the study of Clark et al.[24], that included 345 patients the DFS at 5 years was somewhat more than twice as high in aneuploid tumours (12% versus 26%, p=0.02).

We did not find any statistically significant relation between outcome parameters and S-phase fraction. Published data on this relationship show more disparity than the results of ploidy determination[20,24,28,44,45]. The problems inherent to a proper evaluation of the prognostic importance of SPF can best be discussed in relation to the results obtained in two other almost equally large studies in node-negative patients[22,24]. Clark et al.[24] like us did not find a relationship between outcome parameters and SPF in an univariate analysis of his entire cohort. He did not include SPF in the multivariate analysis. Instead, SPF was found to have a significant prognostic impact in the group with diploid tumours. Given the good prognosis of this group, however, this conclusion was based on only 15 events, 10/97 in the low and 5/15 in the high SPF subgroups. In our 137 diploid patients 14 events have occurred after 5 years with no differences between the low and high SPF subgroups. It is clear, that with such low numbers of events conclusions on the role of SPF to predict recurrence in diploid patients, should be drawn with great caution.

Sigurdsson et al.[37] reported SPF to have a major prognostic impact in univariate and multivariate analyses and in diploid as well as aneuploid populations. One major difference between our and Clark's study is, that they were able to determine the SPF in 94% of their 305 specimens subjected to DNA FCM even without the application of software that is sometimes used to unravel overlapping populations with different degrees of ploidy. We found this impossible in 110/300 (36%) of cases as did Clark et al.[24] in 92/345 (27%) of their histograms. We maintained these patients as missing cases in our analysis and found their prognosis favourable. This suggests, that many of these patients, if analyzed by Sigurdsson et al., would have been classified as to belong to the low SPF group and then have contributed substantially to the significantly better prognosis associated with this group. This is substantiated by the observed distribution of the patients over the less or more than 7% SPF groups. Whereas in both studies an identical 51% of cases had a SPF$\geq$7%, Sigurdsson et al. found 42% to have $\leq$7% and the rest impossible to determine, whereas these figures were 21% and 29% respectively in our study.

Based on these data we feel that the results of studies on the relation between SPF and prognosis heavily depend on what has been done with histograms showing an overlapping aneuploid population of small size. If these patients are considered inevaluable, such as done by us and Clark et al.[24], the prognostic impact of SPF may be absent or restricted to the group with a diploid tumour. If these patients are considered inevaluable but the so-called aneuploid fraction (relative proportion of aneuploid populations) is entered in the analysis, the latter will become a significant prognosticator and SPF again is negative. This has been shown to be the case by Gnant et al.[49]. Finally, some investigators feel that a SPF can be determined in those cases, sometimes with the use of specially developed software[50]. In that case SPF will become a significant prognosticator. Whether this then really indicates an association between proliferation and prognosis or is mainly indicative of the presence of a low aneuploid fraction is at present unknown. Studies using the thymidine labelling index in node-negative breast

cancer patients certainly indicate, that with this measure of DNA synthesis a subgroup with a high chance of relapse (40%) in the first five years can be identified[16,17]. The final answer on the relative contribution of ploidy and proliferation to the prognosis of these patients awaits the evaluation of techniques that combine the specific measurements of proliferation-associated markers and chromosomal abnormalities[51].

Age below 51 was a prognostic factor for recurrence which was lost in multivariate analysis. In a large population-based study in Sweden, Adami et al.[52] found the best prognosis in women between 45 and 49 years old with both younger and older patients having a poorer survival even after 5 years. In that study, however, patients were not stratified for stage. Sigurdsson et al.[37] also found patients under 50 to have a higher rate of recurrent disease but this factor was lost in their multivariate analysis. Several factors may explain this discrepancy, including the lower relative proportion of patients under 51 (24% versus 40% in our study), the restriction of the multivariate analysis to only the 250 patients with complete data and their observed correlation between age and SPF.

Taken together we found ploidy (diploid versus aneuploid/multiploid) and ER (cut-off points 100 and 400) to be the most important independent prognosticators in patients with node-negative pT_1-pT_3 breast cancer. A main advantage of these 2 factors is, that they can be assessed with a minimum of subjectivity or modelling of data. Multivariate analysis indicates ploidy and steroid-receptor content as independent prognosticators. Our data indicate, however, that with the currently available methods we are not able to develop models that predict prognosis in a consistent and clinically reliable way.

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Chapter V

THE PROGNOSTIC AND PREDICTIVE VALUE OF FLOW CYTOMETRY IN NODE-POSITIVE BREAST CANCER PATIENTS

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THE PROGNOSTIC AND PREDICTIVE VALUE OF FLOW CYTOMETRY IN NODE-POSITIVE BREAST CANCER PATIENTS

ABSTRACT

Introduction
DNA flow cytometry derived parameters (ploidy and S-phase fraction (SPF)) may provide important information on the prognosis for relapse and survival of node-positive breast cancer patients. Results have been equivocal, however, which may partly be due to inhomogeneity of studied populations and short follow-up.

Aim
We investigated the prognostic significance of ploidy and SPF in a homogeneously treated axillary node-positive breast cancer population (n=320), postoperatively treated with chemotherapy with or without medroxyprogesterone acetate. The median follow-up time is 7 years.

Results
Age, receptor status and most importantly axillary node status were the only independent prognostic variables in the multivariate analysis. Aneuploidy was a factor predicting poor prognosis in the univariate analysis but, as SPF, was not independently important. However, in the multivariate analysis, patients with aneuploid breast cancers have a significant higher risk for distant metastases (RR 3.59) and tumour related death (RR 2.56) when treated with chemo-hormonal adjuvant treatment if compared to patients with diploid tumours. This was due to the very favourable prognosis of this latter group, also if compared to patients not treated with MPA.

Conclusion
Ploidy and SPF are not independent prognostic factors in this large study node-positive breast cancer patients and should not routinely be analyzed in this population. Ploidy, however, may predict whether a patient would benefit from the addition of endocrine to cytotoxic adjuvant treatment.

INTRODUCTION

Ideally, the strategy for the treatment of breast cancer depends on information on the biological behaviour of the individual tumours. A number of potential prognostic factors have been identified and examined for their ability to predict for outcome of this disease. The most important prognostic indicator is the presence or absence of breast cancer cells in the axillary lymph nodes[1]. Also, tumour size[2,3], histological and nuclear grading and steroid hormone receptor status[4-7] are important prognostic factors which may predict for the clinical outcome of the individual patient.

DNA flow cytometry has been used to predict the clinical outcome of patients with breast cancer. Several studies have been published within the last years, comparing ploidy and S-phase fraction (SPF) with important clinical parameters in node-positive breast cancer patients. The question whether these data provide independent prognostic information on survival or relapse, was answered with conflicting results[8-17], mainly because of relatively small numbers of patients or short follow-up and heterogeneous patient populations.
The aim of this study was to investigate in a large cohort of uniformly staged and treated patients with a median follow-up of 7 years, the prognostic significance of DNA-ploidy and SPF for the clinical outcome in axillary node-positive breast cancer patients. These patients were part of a clinical trial which included locoregional treatment followed by adjuvant therapy. Univariate and multivariate analysis were performed to identify groups of patients with high and low risks for relapse or death from breast cancer.

PATIENTS AND METHODS

Patients
Three hundred and twenty patients with axillary node-positive breast cancer (pT_{1-3}, N_1, M_0) entered a prospective randomized clinical trial between June 1982 and July 1987, analysing the value of adjuvant hormonal treatment in addition to standard chemotherapy. Excluded were patients over 70 years of age, patients with previous malignancies except non-melanoma skin cancer and optimally treated stage Ia cervical cancer. Patients were registered and randomized preoperatively and stratified by T-, N-, menopausal and steroid-receptor status. Surgical treatment consisted either of conservative breast surgery with axillary node dissection (n=45), modified radical mastectomy with axillary node dissection (n=268) or radical mastectomy (n=2). From 5 patients the surgical procedure could not be recovered from the records. Postoperative locoregional irradiation was given in patients with centrally or medially localized breast cancer (parasternal radiation field) (n=46) and in patients with microscopically irradical operation (chest wall radiation) (n=18). All 45 patients who underwent conservative breast surgery had complete radiation of the breast with a boost at the lumpectomy site. Patients with tumour containing lymph nodes were treated with adjuvant therapy according to the allocated treatment of the trial (see further). The clinical characteristics of the patients are shown in table I. The median follow-up time of the 320 patients was 84 months, as of their last recorded follow-up evaluation; 171 patients were alive (53.4%) and 143 patients were free of recurrence (44.7%).
In 91 (28.4%) of the patients a pathologically T_1 (pT_1) tumour was diagnosed; in 199 (62.2%) of the women the tumour was classified as pT_2 whereas in 9.4%[30] of the patients a pT_3 tumour was diagnosed.

The distribution of the number of involved nodes in these patients was as follows: 183 women (57.2%) had 1-3 axillary nodes containing breast cancer, 108 women (33.8%) 4-9 tumour containing nodes, and 29 women (9.0%) $\geq$10 pathological nodes.

Table I Patient characteristics of node-positive breast cancer patients.

Parameter	Number of patients	Percentage
Size		
pT_1	91	28.4
pT_{2-3}	229	71.6
Ploidy		
diploid	89	27.8
aneupl/multipl	203	63.4
"no data"	28	8.8
Histology		
infiltrating ductal ca.	282	88.1
infiltrating lobular	17	5.3
miscellaneous	21	7.6
Number of positive nodes		
1-3	183	57.2
4-9	108	33.8
>9	29	9.0
S-phase fraction		
≤8%	88	27.5
>8%	70	21.9
"no data"	162	50.6
Progesterone-receptor		
≤60	169	52.8
>60	80	25.0
missing values	71	22.2
Oestrogen-receptor		
≤10	119	37.2
>10	186	58.1
missing values	15	5.7
Age distributions		
≤50 years	134	41.9
>50 years	186	58.1

From 249 (77.8%) women the progesterone-receptor (PgR) of the primary tumour was known whereas in 305 (95.3%) of the cases the oestrogen-receptor (ER) could be calculated. This discrepancy in numbers is caused by later availability of PgR determination.

Adjuvant treatment of the node-positive patients and follow-up

Patients were randomly allocated into two groups: A. chemotherapy consisting of cyclophosphamide 500 mg/m^2, doxorubicin 40 mg/m^2, and 5-fluorouracil 500 mg/m^2 on day 1 q 4 weeks for a total of six cycles of chemotherapy; B: the same chemotherapy schedule as in A in combination with medroxyprogesterone acetate (MPA) daily 500 mg intramuscularly (i.m.) for 28 days, followed by 500 mg i.m. twice a week for 5 months. Follow-up included a history and physical examinations, blood counts, glutamyl transferase (gamma-GTP), lactate dehydrogenase (LDH), calcium, erythrocyte

sedimentation rate and carcino-embryogenic antigen (CEA). Chest radiographs and mammography were performed annually.

Histological examinations and flow cytometry

The histologic grade was determined by the method of Bloom and Richardson[18]. Oestrogen- and progesterone-receptor contents were assayed with the dextran-coated charcoal method, Scatchard analysis and calculation according to the method of Lowry[19,20].

Flow cytometric determination of DNA levels was performed in nuclei isolated from paraffin-embedded tissue[9,21]. Fifty μm sections were cut from formalin fixed paraffin embedded tissue blocks containing a representative sample of the primary tumour. Five μm sections were cut for histological control to make sure that the 50 μm sections contained tumour. DNA content was measured by the method of Vindelǿv[22]. Tumours with a single G1 peak were considered to be diploid, whereas evidence of an additional G1 peak indicated aneuploidy. DNA index (DI) was calculated as the ratio of aneuploid to diploid G1/0 peak channel. Histograms with coefficients of variation (CV) less than 8% were considered of good quality. Histograms with CV > 8% were classified as not interpretable. The proliferative activity (SPF) was planimetrically calculated by counting the number of cells between the inclination points of the descending G1 peak and the ascending G2/M peak[9,23]. The SPF calculations were performed by two individuals, independently from each other. In case of discrepancy in calculations the results were middled. In cases of less than 30% admixture of diploid cells, the percentage of aneuploid S-phase cells was calculated without corrections for the presence of diploid S- and G2/M phase cells. In case of more than 30% admixture diploid cells in overlapping of diploid and hyperdiploid histograms the percentage of S-phase cells was not calculated. When ploidy status could not be determined because of poor quality of the sample, these histograms were considered uninterpretable.

After descriptive analysis the cutoff levels for the proportion of S-phase cells were set at $\leq 8\%$, and $>8\%$ in order to define two groups, with low and high SPF respectively.

Statistical analysis

To investigate the prognostic significance of the clinical parameters included in the study, Kaplan-Meier estimates of survival curves, logrank tests for comparing two or more groups and the Cox proportional-hazards regression model were used. All analyses were done using the statistical packages SAS (SAS Institute Inc., Cary, NC, USA) and S-PLUS (Statistical Sciences Europe, Oxford, UK).

First routine univariate analyses were done to compare survival curves using quartile, quintile and accepted clinical cutoff points for the prognostic variables with respect to the end points 'overall survival' (OS) and 'distant metastasis free survival' (DMFS).

Secondly all prognostic variables were included in a Cox regression analysis with respect to these endpoints. For every variable the assumptions of the Cox regression model, i.e. proportional hazards and log-linearity of effect, were checked.

The proportional hazards assumption was checked through different graphical plots and using tests based on Schoenfeld residuals. Log-linearity of effects was checked through generalized additive modelling and smoothing of partial residual plots.

Influence diagnostics and residual plots were also used to check for undue dependence of

the 'definite' models on the influence of a subset of patients.
To examine a possible difference in prognostic value of the clinical parameters between the MPA-positive and the MPA-negative treatment groups, all interaction terms between MPA and the clinical parameters were included in the model. Backward elimination was used to retain only those interaction terms suggestive of systematic prognostic differences between MPA treated and non-MPA treated patients.
With regard to both OS and DMFS there was a definite suggestion of non-proportionality for the effect of oestrogen-receptor content and of a possible non-linearity of effect for age, S-phase percentage, progesterone-receptor content, oestrogen-receptor content and number of positive axillary nodes. The non-linearities suggested by the analyses could in all cases adequately be simplified by taking cut-off points: oestrogen-receptor content at 10 fmol/mg protein, progesterone-receptor content at 60 fmol/mg protein, number of positive axillary nodes at 3 and 9, age at 50 and S-phase at 8%. The non-proportionality of oestrogen-receptor content was resolved by including a time dependent effect; the difference in effect between ER-content ≤ 10 and ER > 10 seemed to diminish between about 4 to 5 years after diagnoses for DMFS and at approximately 4 to 6 years after diagnoses for OS.
The p-values derived from multivariate analyses are the end result of data exploration and should not be interpreted as direct tests of a hypothesis of no prognostic effect. The Kaplan-Meier curves and logrank tests presented for the prognostic variables are based on the cut-off points used in the multivariate analyses.
Given the for this analyses relatively small number of patients and events we did not attempt split-sample validation or correction for overfitting.
Correlation of covariates was calculated using Kendall's rank correlation τau. Age and ploidy were correlated: older women (>50 years) had a higher percentage aneuploid tumours compared to women ≤ 50 years ($r=0.279$, $p=0.003$). ER and PgR were correlated with aneuploidy (r-values respectively 0.241 and 0.232, p-values respectively 0.01 and 0.02).
No correlation was found between SPF and number of axillary nodes or between SPF and other combinations of variables.

RESULTS

From the 320 patients in 12 (3.7%) no tumour specimen was available and in 16 (5%) the histogram could not be interpreted. The main characteristics of these cases did not differ significantly from those patients with interpretable histograms, nor was the RFS or OS different for these 28 patients. The following analyses were related to all 320 patients with axillary node-positive breast cancer. Of the analyzed patients 27.8% were diploid and 63.4% aneuploid or multiploid. In 158 (49.4%) of these patients it was possible to determine the SPF. Of all tumour specimens 27.5% had an SPF $\leq 8\%$, 21.9% had an SPF $> 8\%$, and 50.6% was not analyzable.

Univariate analysis
Univariate analysis performed with all the covariates listed in table I revealed that ploidy status was able to distinguish between patient groups at high and low risk for overall survival (OS) (p=0.03) and distant metastases free survival (DMFS)(p=0.04)(figure 1, 2).

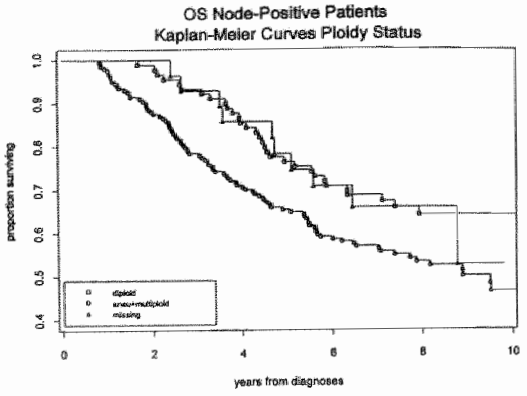

Figure 1 Kaplan-Meier overall survival (OS) curves in node-positive breast cancer patients. Diploid breast cancer patients have a lower risk for dying (p=0.03).

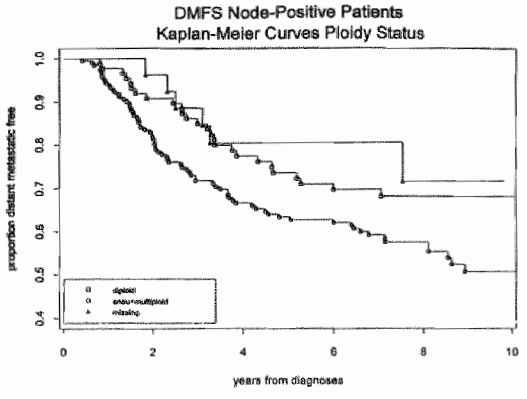

Figure 2 Kaplan-Meier distant metastases free survival (DMFS) curves in node-positive breast cancer patients. Diploid patients have a lower risk for distant metastases (p=0.04).

SPF was not able to distinguish between a high and a low risk group. Kaplan-Meier curves for OS and DMFS showed a statistically not significant higher risk for SPF stratum $\geq 8\%$. Other significant variables in univariate analyses for DMFS and OS were pT, PgR status (cut-off point 60), age and number of tumour containing axillary nodes.

Table I Univariate analysis of prognostic variables in 320 node-positive breast cancer patients (pT_{1-3}) with a median follow-up of 7 years.

Parameter	No pts.	DMFS No events obs	exp	p-value	OS No events obs	exp	p-value
Ploidy							
diploid	89	28	36.2		31	43.4	
aneupl&multipl	203	77	64.3	0.04	95	80.1	0.03
"no data"	28	6	10.5		10	12.6	
Size							
pT_1	91	23	36.4		28	43.0	
pT_{2-3}	229	88	74.6	0.007	108	92.9	0.006
ER status							
≤10	119	32	38.5		53	45.61	
>10	186	72	66.9	0.40	78	53.6	0.36
missing	15	7	5.5		5	6.8	
PgR status							
≤60	169	65	51.1		85	65.2	
>60	80	19	32.2	0.01	23	38.3	0.002
missing	71	27	27.7		28	32.5	
Age							
≤50	134	33	51.1		42	61.1	
>50	186	78	59.9	0.001	94	74.9	0.001
SPF							
≤8	88	31	34.2		34	41.7	
>8	70	25	21.7	0.67	30	27.7	0.36
"no data"	162	55	55.03		72	66.6	
Number axillary nodes							
1-3	183	45	73.04		50	87.3	
4-9	108	49	32.76	<0.001	61	41.7	<0.001
≥10	29	17	5.2		25	7.08	

Multivariate analysis

Cox proportional hazards regression with respect to DMFS revealed that age, PgR, ER and the number of tumour containing axillary nodes were independent prognostic factors (table II). Ploidy was not an independent prognostic factor. However, if MPA treatment was also taken into account it appeared that MPA-positive aneuploid and multiploid patients had a hazard ratio (RR) of 3.59 (95% CI 0.74-17.29) for DMFS and of 2.56 (95% CI 1.24-5.31) for OS as compared to diploid patients (p=0.004 and 0.02 respectively) (table II). For MPA-negative patients hazard ratio's close to 1 (0.81 for DMFS and 1.01 for OS) were observed. With this finding from the multivariate analysis in mind, Kaplan-Meier curves were constructed showing a significant lower risk of relapse and death for diploid, MPA-treated patients as compared to the three other groups (figure 3). Apparently diploidy is a positive predictor for a MPA treatment effect.

Table II Multivariate analysis (Cox proportional hazards regression) of prognostic covariates of survival in 320 node-positive (pT_{1-3}) breast cancer patients with a median follow-up of 7 years.

Parameter	DMFS ß	DMFS p-value	DMFS RR	OS ß	OS p-value	OS RR
MPA	-0.82	0.19	0.440	-0.69	0.05	0.502
pT_{2-3}	-0.25	0.43	0.778	0.12	0.59	1.128
an+multipl	-0.22	0.54	0.805	0.01	0.96	1.015
ER ≤ 10 < 5 yr	0.57	0.03	1.776	1.05	0.0001	2.850
PgR ≤ 60	0.62	0.02	1.857	0.50	0.04	1.638
Age > 50	1.04	0.002	2.855	0.45	0.01	1.572
N axill.nodes						
4-9	0.64	0.003	1.910	0.77	0.0002	2.163
≥ 10	1.95	0.0001	7.005	2.01	0.0001	7.472
SPF > 8	0.38	0.33	1.462	0.28	0.36	1.320
MPA an+multi	1.49	0.004	3.591	0.93	0.02	2.555
MPA age > 50	-1.03	0.02	0.357	-0.63	0.11	0.535
MPA SPF > 8	-1.27	0.04	0.408	-0.99	0.03	0.493
MPA pT_{2-3}	0.97	0.04	2.646			

pT_{2-3} : pathological stage T_2 and T_3
MPA an+multi : patients treated with medroxyprogesterone acetate, with aneuploid and multiploid tumours.
ER ≤ 10 < 5 yr : oestrogen-receptor ≤20 fmol/mg protein; follow-up less than 5 years after diagnosis
PgR ≤ 60 : progesterone-receptor ≤60 fmol/mg protein
N axill.nodes : number of tumour containing axillary nodes
SPF : S-phase fraction
ß : parameter estimate
RR : risk ratio

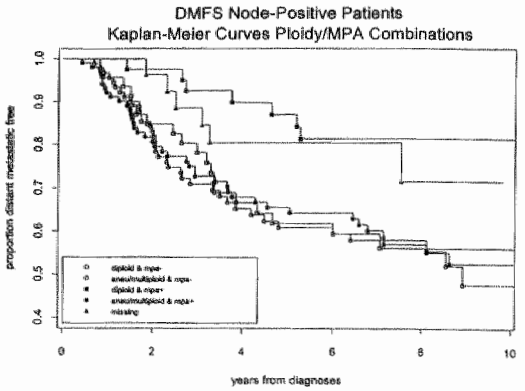

Figure 3 Kaplan-Meier DMFS curves in node-positive breast cancer patients combining ploidy and MPA treatment. Diploid patients treated with MPA have a lower risk for distant metastases.

SPF was not a significant prognostic factor in the multivariate analysis. Treatment-related analyses suggested some predictive effect for the treatment with MPA. The RR of patients with MPA-treatment and SPF≥8% was 0.41 (95% CI 0.008-2.1) for DMFS and 0.49 (95% CI 0.19-1.27) for OS compared to patients with SPF<8% (table II). Without MPA treatment the RR's were slightly over 1 (not shown). Kaplan-Meier curves suggest that MPA improves the prognosis of the SPF≥8% group, which in itself is doing rather poorly (figure 4). Interpretation of these data is hampered by the relatively large group of patients without SPF data.

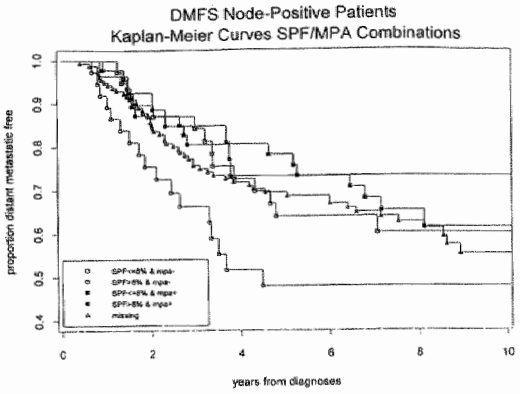

Figure 4 Kaplan-Meier DMFS curves in node-positive breast cancer patients combining SPF and MPA treatment. Patients with SPF>8% treated with MPA have a lower risk for distant metastases

DISCUSSION

The main result of our study is that in node-positive breast cancer patients treated with adjuvant chemotherapy, FCM derived parameters have no independent prognostic effect after correction of stage, age and receptor status but rather may be predictive factors for the effects of additive high-dose treatment with a progestagen. This important result may be the start for defining new roles for aneuploidy and cell kinetics and also may in part explain conflicting results from earlier studies, that used a variety of cytotoxic and hormonal adjuvant treatments.

In the literature diploidy of breast tumours has been reported to be a favourable prognostic indicator using univariate analysis[8,10-12,24-30]. However, some investigators did not detect a significant correlation between ploidy levels and prognosis[13,31,32] whereas in other studies this relationship was limited to node-positive patients[8,10,28] or has lost significance in multivariate analysis[10,33,34]. Our study shows that diploid patients treated with chemotherapy combined with MPA have a significant lower risk compared to non-diploid patients treated with the same adjuvant therapy and compared to the diploid

group of patients treated with adjuvant chemotherapy only. This suggests a predictive rather than a prognostic role of ploidy for breast cancer patients who are being treated with combinations of cytotoxic and endocrine treatment.

One explanation of the finding, that diploidy predicts a positive adjuvant effect of MPA could be, that diploidy serves as a surrogate marker for ER or PgR-positivity. Indeed we observed a correlation between ER and PgR status on one hand and ploidy on the other hand. This has also been reported in other studies. However, the correlation in our study was only weak with correlation coefficients below 2.5. Moreover, in the Cox regression modelling all interaction terms between MPA treatment and clinical variable were included. Ploidy-related rather than receptor-related differences were picked-up as predictors for prognosis in a MPA-treatment dependent fashion.

Another explanation may be, that aneuploid tumours have lost their ability to respond to MPA treatment due to other mechanisms than the loss of receptor. MPA has been shown to modify multidrug resistance[35]. The CAF regimen contains one cytotoxic agent, doxorubicin, that is sensitive to the activity of the MDR-1-associated p-glycoprotein[36]. Patients with MDR-1-positivity can be expected to become more sensitive to CAF if the mechanism, by which MPA can revert MDR is intact. Clearly, diploid and aneuploid tumours are not equally sensitive to CAF as evidenced by the difference in prognosis in the univariate analysis. Studies relating CAF adjuvant effects to MDR status are needed to determine, whether this is due to differences in MDR-positivity. The RR for DMFS and OS is close to 1 for aneuploid versus diploid tumours in the not MPA-treated group, suggesting that MDR per se does not play a major role in the differences, observed once MPA is added. This latter, predictive effect could however be explained by assuming that not the MDR-status itself, but the MDR modulating effect of MPA is different in diploid versus aneuploid cells. Further studies are needed to explore this intriguing possibility.

The role of the SPF in predicting the prognosis and therapy effect is probably small, if any. In most studies a high SPF value was found to be associated with a shorter DFS and/or OS time[8,12,13,30,37-39]. In some studies the SPF turned out to be a more powerful prognostic factor than DNA ploidy[12,37,38,40], however, in other reports the independent prognostic value of SPF was lost when the histological grade was included in the analysis[13,37,41]. A number of studies did not include (because of unknown reasons) SPF in their flow cytometric investigation[10,12,28,42-46]. If MPA treatment is taken into account it appears, that particularly patients with SPF $\geq$ 8% benefit from this treatment. This should not be taken to indicate, that high proliferation predicts MPA sensitivity. The results were obtained against a background of treatment with CAF for all patients. The results therefore could also be related to differential sensitivities of high and low proliferative cells to chemotherapy with equal sensitivity to MPA. The survival curves suggest that CAF alone is not very active in highly proliferative tumours. Whether this is true can only be demonstrated from studies with a no treatment arm.

In this study ploidy is independently correlated with age, ER and PgR content. No correlation can be observed between ploidy and SPF or between SPF and steroid-

receptor content.
In the literature discrepancies are described in correlation between ploidy and axillary nodal status[29,42,55,47-49], and between ploidy and ER-status[10,11,28,42,47,48,50-53].
Reports on correlations of nodal status with SPF are conflicting as well[47,49-52]. More unequivocal results are found in reports about correlations between SPF and ploidy[41,48,50,54].
Although in our study as in others the number of tumour containing axillary nodes, steroid-receptor status, and age are useful prognostic factors in node-positive breast cancer patients, individual decision making and individualizing adjuvant therapy remain difficult. Parameters derived from DNA flow cytometry do not have a major contribution in this respect. Additional reliable prognostic factors, studied prospectively in a homogenous patient population, are needed to individualize adjuvant therapy for node-positive breast cancer patients.

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Chapter VI

PLOIDY AND S-PHASE FRACTIONS (SPF) OF PRIMARY BREAST CANCERS AND THEIR NODAL METASTASES

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PLOIDY AND S-PHASE FRACTIONS (SPF) OF PRIMARY BREAST CANCERS AND THEIR NODAL METASTASES

SUMMARY

Ninety-one cases of primary breast cancers and their nodal metastases were examined with DNA flow cytometry. No differences were found between the stemline distributions in the primary tumours and nodal metastases. At both sites stemlines clustered around a DNA index of 1.0 (33-40% of cases) and 1.8.
The mean S-phase fractions were 7.9 in primary tumours versus 5.6% in nodal metastases ($p=0.02$); this difference was also observed if the analysis was restricted to cases with DNA aneuploidy at both sites (10.2 versus 7.6%, $p=0.04$). Our results indicate that axillary nodal ploidy and proliferation reflect primary tumour characteristics rather than display changes associated with selection during the lymphatic metastatic process. Lymph nodes may have a suppressive effect on the proliferation of tumour cells.

INTRODUCTION

In primary breast cancer DNA flowcytometry (FCM) provides reproducible information regarding DNA content (ploidy) and proliferation (S-phase fraction or SPF). In most studies these tumour-associated characteristics were found to predict for prognosis[1,2,3,4,5] although this has not been universally observed or was limited to subsets of patients[6,7,8,9]. One factor that may contribute to the inconsistency of these results is intratumour heterogeneity. Tumour containing axillary lymph nodes, because of the occurrence of selection towards metastatic subpopulations, may provide different and prognostically more relevant information regarding ploidy and proliferation characteristics. As a first step towards answering this question we performed DNA FCM on archival material from 91 primary breast cancers and their axillary nodal metastases and compared ploidy and proliferation at these two sites.

MATERIAL AND METHODS

Ninety-one cases with histologically proven node-positive primary breast cancer were examined. They represent all consecutive cases that were contributed by one institution to a multicenter trial of the addition of medroxyprogesterone acetate to adjuvant chemotherapy with cyclophosphamide, doxorubicin and 5-fluorouracil (FAC)[10]. Fifty μm sections were cut from formalin-fixed paraffin-embedded tissue blocks containing at least one sample of the primary tumour and one sample of a nodal metastasis. Four μm cut in

continuity with the 50 μm sections were Haematoxylin-Eosin (H-E) stained and were used for histologic control. The analysis was restricted to axillary nodes of which at least 25% of the cut surface as visualized by H-E staining was occupied with tumour. This percentage was visually evaluated on H-E slide. Infiltrates of lymphocytes in the tumour were not taken into account. Single nuclear suspensions were made from the 50 μm sections according to a method described[11]. In brief, the sections were placed in 10 ml centrifuge tubes and dewaxed in 6 ml xylene for 15 minutes at room temperature. Rehydration was performed in a sequence of 100%, 96%, 70% and 50% ethanol with centrifugation and decantation of the supernatant after each step. The sections were washed in 5 ml phosphate-buffered saline (PBS, pH 7.4). Three ml of 0.25% trypsin (DIFCO) in citrate buffer (3 μm trisodium citrate, 0.1% nonidet P40, 1.5 mM spermine tetrachloride, 0.5 mM Tris pH 7.6) was added and the tubes were incubated overnight at 37°C. After vortex mixing the sample was filtered through a 50 μm nylon mesh. Approximately 2-3 x 10^6 nuclei were stained according to the method of Vindelöv et al.[11]

FLOW CYTOMETRY

Cellular DNA content was measured on a FACS IV Cell Sorter (Becton and Dickinson, Sunnyvale, CA) equipped with a 4W Argon laser (Spectra Physics, model 164-01) operating at 488 nm. Histograms of 10^4 cells were recorded. Deviations in cellular DNA content (aneuploidy) were expressed as the DNA index (DI). The DI was calculated as the ratio of aneuploid to diploid G1/O peak channel; in case of ≥ 2 peaks the left-sided was always considered as diploid. DNA indices between 0.9 and 1.1 are considered to be diploid; between 1.9 and 2.1 tetraploid; >2.1 hypertetraploid and the remaining DNA indices are considered aneuploid. Histograms with coefficients of variation less than 8% were considered of good quality. The proliferative activity was calculated by visually marking the area between the right inclination of the G1 and the left inclination of the G2M peak and counting the number of cells between these markings. The visually marked SPF calculation was performed by two individuals separately. In around 60% of all DNA histograms SPF can be calculated. In cases of less than 30% admixture of diploid cells, the percentage of aneuploid S-phase cells was calculated without corrections for presence of diploid S- and G2/M phase cells. In case of more than 30% admixture of diploid cells and overlap of diploid and hyperdiploid histograms the percentage of S-phase cells was not calculated.

STATISTICS

S-phase fractions from primary tumours and axillary metastases were compared using a one way anova or, if there was a concordant DI at both sites, a paired t-test.

RESULTS

Results from ploidy analyses in 91 cases of primary breast cancer are shown in figure 1 as the frequency distributions of DNA stemlines from primary tumours and their nodal metastases. The diploidy rate was slightly higher in lymph nodes (40% versus 33%, $p<0.05$) and at both sites the DNA indices clustered in the 1.0 and 1.8 range. If analyzed for DNA-index correlations at the level of individual patients only 39/91 (43%) of cases showed identical DNA ploidy clones in the primary tumour and their nodal metastases. This result has to be interpreted, however, against our finding of a 41% sampling variability in DNA ploidy from different biopsies of the same primary tumour.

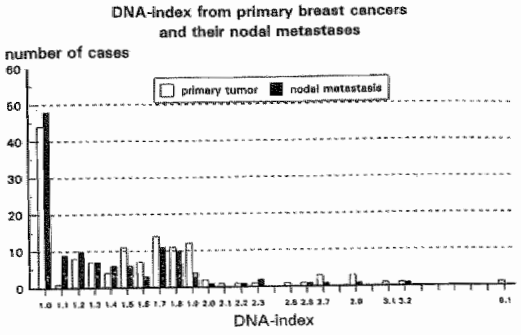

Figure 1 Frequency distribution of DNA stemlines from primary tumours and their nodal metastases.

S-phase fractions (SPF) could be determined in all diploid cases (n=20), in 6 cases with a diploid primary tumour and aneuploid nodal metastases, 6 cases with aneuploid primary and diploid node, 6 cases with concordant aneuploidy at both sites and 2 cases with discordant aneuploidy. The frequency distribution is shown in figure 2 for primary tumours and metastatic axillary nodes. Since these data suggest an overall lower nodal SPF as compared to the primary tumour, we further analyzed this relationship in more detail; the results are presented in table I. A significant difference was observed with the SPF of the primary tumour exceeding that of the metastatic node with 2.3%. If the analysis was restricted to cases with concordant ploidy (diploid or aneuploid at both sites) the differences remained of the same order of magnitude and were significant, even in the case of aneuploid tumours with a very small sample size (table I). For the interpretation of these data it is important to know the background of SPF of not tumour-containing lymph nodes. Therefore, 13 samples from node-negative breast cancer patients were also analyzed. Their mean SPF was 4.8% (range: 2.3-8.7; SEM 1.6).

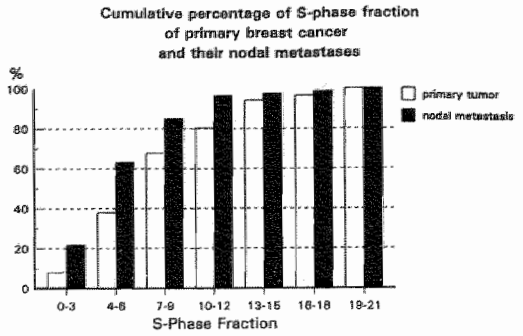

Figure 2 Cumulative percentage of S-phase fraction of primary breast cancer and their nodal metastases.

Table I S-phase Fractions in Primary Breast Cancer and Nodal Metastases: Comparison.

	No	SPF (%) primary	node	p-value**	correlation coeff.
all cases (SEM)*	40	7.86 (0.70)	5.69 (0.60)	<0.01	0.5403 ($p<0.05$)
cases with DI=1.0	26	6.83 (0.61)	4.63 (0.64)	<0.01	0.5584 ($p<0.05$)
cases with DI#1.0	14	10.46 (1.908)*	7.58 (1.40)*	<0.04	0.7242 ($p<0.05$)

* SPF determination possible on all diploid and 20/45 (44%) of aneuploid cases.
** paired t-test for cases with concordant DNA-index

Despite the differences in SPF between primary tumours and nodal metastases a significant correlation between the SPF at the two sites could be demonstrated, in particular if the analysis was limited to cases with concordant stemlines. This is shown in figure 3a for diploid/diploid stemlines and in figure 3b for aneuploid/aneuploid stemlines. In both instances significant correlation coefficients were obtained of 0.56 and 0.72 respectively ($p<0.05$). Taken together these data indicate, that the proliferative characteristics of a breast tumour are maintained in the process of regional nodal metastasis albeit at a lower level of activity.

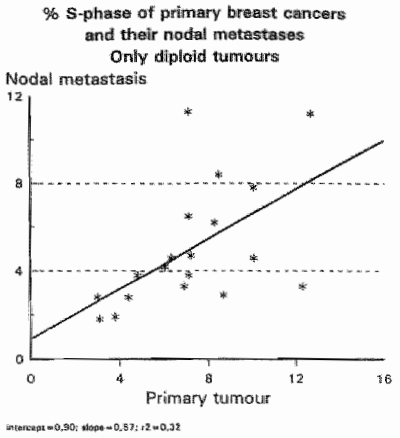

Figure 3a Correlation between SPF of primary tumours and tumour containing lymph nodes in diploid tumours

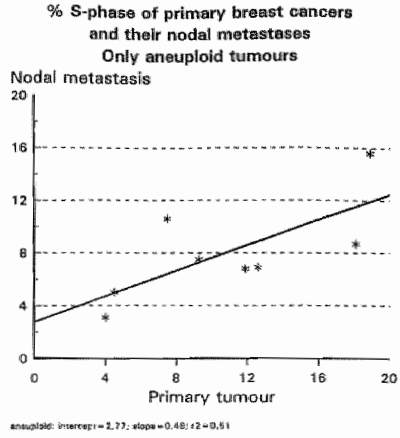

Figure 3b Correlation between SPF of primary tumours and tumour containing lymph nodes in aneuploid tumours

DISCUSSION

In this large series of patients with primary breast cancer and axillary nodal metastases no differences could be observed between the distribution of stemlines in primary tumours and their nodal metastases. Similar results have been described by other investigators[12-16]. At the level of individual patients complete concordance of stemlines between primary tumour and axillary nodes was observed in only 43% of cases. This figure however is difficult to interpret because of considerable sampling variability. Beerman et al.[16] found intra-tumour heterogeneity (and therefore sampling variability) in 80% of primaries and 66% of axillary nodal metastases; with the examination of 2 rather

than 1 nodal sample we observed a 41% chance of finding an additional stemline. Therefore the difference in DNA ploidy between individual primary tumours and their nodal metastases can well be caused by intra-tumour heterogeneity. The similar stemline distribution profiles at both sites certainly suggest that if clonal selection occurs during the metastatic process, this does not result in clonal restriction as expressed in flow cytometrically measured DNA content.

In contrast to others[12,13] we found the nodal SPF to be significantly lower than the SPF of the corresponding primary tumours. One obvious explanation of this finding would be dilution effect due to the admixture with non-malignant nodal cells (e.g. lymphocytes) with a low proliferation rate. We consider it unlikely that this is the sole explanation for two reasons. Firstly, the difference was also apparent if the analysis was restricted to cases with aneuploid stemlines in both the primary tumour and their axillary nodes. In fact, the difference was even somewhat greater than with diploid tumours and probably only less significant ($p=0.04$) because of the small sample size. It is difficult to imagine how a dilution effect may explain the SPF difference in this circumstance. Secondly, the mean SPF of tumour-negative axillary nodes equalled that of tumour-positive nodes in cases of diploid tumours. Most of the metastatic nodes contained a considerable amount of tumour; we selected nodes with at least 25% of the cut surface consisting of tumour and often this percentage was considerably higher. Therefore, the mixture of non-malignant lymph node cells with a SPF of 4.8% and malignant diploid cells with a SPF of 6.8% (as is the case in the primary tumours with a diploid stemline) should have resulted in a higher SPF in the metastatic nodes than the observed 4.6%. This again points clearly to the fact that nodal tumour cells indeed have a lower proliferation rate as compared to their primary counterparts. Obviously a final demonstration of this phenomenon would require the presence of a second marker, that would allow to restrict the measurement of proliferation to clearly defined malignant cells. Such an approach could not be applied in our study that makes use of quantitative DNA measurements on isolated nuclei but may become feasible by combining other proliferation markers with phenotypic markers of malignancy[19].

We did not apply one of the recently developed computer programs to calculate the SPF. Such programs use mathematical models to determine the SPF, in some cases account for the presence of debris and may give results in cases that in the method of manual gating is considered not suitable for reliable SPF-calculations. Recent comparative investigations have explored the various methodologies[20,21]. In a study of non-Hodgkin's lymphomas the manual gates model yielded an accurate SPF estimate in most cases and was not inferior than computer models with rectangular or polynomial fit and debris subtraction. Also, it is clear from our own experience and from data in the literature that sampling variations by far exceed the variations introduced by the different methods of calculating S-phase fractions in a given histogram. Therefore, computer-aided S-phase analysis has the advantage of greater objectivity but not necessarily of greater accuracy. The choice of the appropriate algorithm depends on the histogram to be analyzed and may be subjective. Manual gating remains an acceptable and reliable alternative.

The nodal SPF, though significantly lower, at the same time correlated significantly with the primary SPF and the distribution profile of the nodal SPF, though shifted to the left, appeared similar. If tumour cells with a low proliferative potential had a higher propensity to metastasize, one would expect the distribution profile to show a more enhanced clustering around lower values and the scatterplots to diverge from a straight line. Clearly neither is the case. It appears, therefore, that the lower nodal SPF is not the result of metastatic selection, but rather is determined by both the inherent proliferative capacity of the primary stemline and the metastatic environment, that in axillary nodes appears to be suppressive for proliferation.

In conclusion, axillary nodal ploidy and proliferation reflect primary tumour characteristics rather than display changes associated with the metastatic process; the lymph node, however, may be suppressive for proliferation. In view of these findings it appears reasonable to hypothesize, that calculation of the proliferative rate of tumour containing axillary lymph nodes will have no prognostic impact over that obtained from the primary tumour. A follow-up study on a larger population is needed to investigate this hypothesis in a more definitive way.

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Chapter VII

SUMMARY AND GENERAL DISCUSSION

SUMMARY AND GENERAL DISCUSSION

INTRODUCTION

Breast cancer is a heterogeneous and complex disease for which in the near future no dramatic improvement in survival is to be expected in terms of current therapeutic concepts. The greatest impact on disease free survival and survival, during the last 30 years, has been achieved with adjuvant treatment with multi-agent chemotherapy, prolonged tamoxifen treatment and the combination of chemo- and endocrine treatment[1,2].

The last decade has witnessed a large increase in the size of biologic information relating to normal and abnormal breast development. The use of molecular techniques in combination with refined methods of histologic assessment have provided information about genetic changes during the development of the malignant process, about factors that influence cell proliferation and about factors that are prognostic of outcome. A number of most widely used factors are summarized in table I. However, despite this surge in new prognostic variables, the therapeutic options now available to a woman who is diagnosed to have breast cancer, have been derived empirically, and in general they have not been refined by the impact of biological factors.

In this study on node-negative and node-positive breast cancer patients, in which diagnosis, staging and loco-regional therapy was performed uniformly, we attempted to relate clinical and biologic properties (pTNM, number of involved axillary nodes, steroid-receptor status, DNA-index, S-phase percentage and age) and aspects of treatment to the subsequent prognosis and response to treatment in human breast cancer. The results will be summarized and discussed in view of their practical applications and significance for further studies.

Table I A number of prognostic indicators in breast cancer

Prognostic Factor	Relevant to Etiology	Guide to Therapy	Reference
Nodal status	No	Yes	Fisher et al.[3]
Tumour size	No	Yes	Neville et al.[4]
Nuclear grade	No	Yes	Neville et al.[4]
Oestrogen and progesterone receptor	Possibly	Yes	Fisher et al.[3]
Ploidy	Possibly	No	O'Reilly&Richards[5]
S-phase fraction	No	Possibly	O'Reilly&Richards[5] Gasparini et al.[6]

ADJUVANT CHEMO-HORMONAL TREATMENT

In Chapter II of the study post-operative chemo-hormonal treatment is compared to chemotherapy alone in node-positive breast cancer patients. Patients ≥60 years showed a significantly longer disease-free survival and overall survival when medroxyprogesterone acetate (MPA) was added to CAF chemotherapy. The beneficial effect may be explained by higher oestrogen-receptor levels (ER) in elderly breast cancer patients. In women ≤40 years the addition of MPA to chemotherapy has a detrimental effect which may be explained by the protective effect of MPA on ovarian function, preventing chemotherapy-induced ovarian ablation. A second explanation of the detrimental effect of the combination CAF with MPA could be a decrease in percentage of S-phase cells, caused by MPA, leading to a reduced effect of chemotherapy.

MPA in high dose added to chemotherapy has a beneficial effect in elderly patients on freedom from distant metastases and overall survival. However, side effects of MPA, especially weight gain and local infection from intramuscular administration are of concern. Since low dose MPA (300 mg p.o.) has the same remission rate in comparison to high dose MPA (900 mg p.o.)[7], a low oral dose could be chosen in an adjuvant treatment schedule in order to ameliorate these side effects. High dose MPA (500-1000 mg p.o. or i.m.) was as effective as tamoxifen in advanced breast cancer in a number of studies[8,9,10]. These data support further exploration of MPA (300-500 mg p.o.) as adjuvant treatment in postmenopausal axillary lymph node-positive breast cancer patients in comparison to tamoxifen 20 mg.

Adjuvant tamoxifen treatment is used post-chemotherapy in a dosage of 20 mg during 2-3 years. Data on MPA adjuvant treatment started after chemotherapy and administered for longer than 6 months are not available. Tamoxifen has interesting other side effects such as on the bone and the cardiovascular system[11,12]. It also reduces the incidence of contralateral breast cancer but may increase the risk for endometrial cancer[13]. This later effect is absent with MPA; recently this drug has been found to be as effective as tamoxifen to prevent contralateral breast cancer[14].

Taken together it certainly is too early to replace anti-oestrogens with progestagens in the adjuvant treatment of node-positive postmenopausal breast cancer patients. If the efficacy of the two approaches is indeed similar, other factors such as oncological and non-oncological side effects become important. Trials comparing long term tamoxifen with short and long term oral progestagens are needed to address these issues.

STEROID-RECEPTOR AND PROGNOSIS

The steroid-receptor status is in literature mostly studied as a dichotomous variable (≤10 fmol/mg protein versus >10). In chapter III oestrogen- (ER) and progesterone-receptor (PgR) are studied as a continuous variable. Node-negative breast cancer patients with ER-contents between 100 and 400 fmol/mg protein have a low risk of recurrence whereas the classical (clinically used) cut-off point of 10 fmol/mg has no significant

prognostic value in this study. Patients with very high ER levels (>400 fmol/mg) do significantly worse, but this phenomenon may be influenced by the low number of patients in this subgroup. These results are time independent and are seen for distant metastasis free survival (DMFS) and overall survival. In node-positive breast cancer patients the ER-status (≤10 or >10 fmol/mg protein) predicts prognosis in a time-dependent fashion, that is in the first 5 years after diagnosis.
The PgR is a time dependent prognostic factor in node-negative patients. Patients with a PgR content >80 fmol/mg protein have more distant metastases and an excess of deaths after a follow-up period of more than 5 years whereas patients with a PgR content ≤80 fmol/mg have a higher risk up to about 5 years after diagnosis. In node-positive patients a cut-off point of 60 shows to be a significant prognosticator meaning that values ≤60 predict for a higher risk of distant metastasis and death. This effect is time-independent.

Given our data and those from the literature it is very difficult to come to a clear and firm conclusion regarding the use of the steroid-receptor status as a prognosticator for patients with operable breast cancer. Two points, however, seem to emerge. The first is the time-dependency of the prognostic information. In our study low ER-levels in node-positive and low PgR-levels in the node-negative patients predict a poorer prognosis for the first 5 years after the diagnosis. This effect disappears or is even counter balanced (in case of PgR) after that time. Similar time-dependent effects have been observed by others[15]. The reasons for this are difficult to dissect. Part may be due to a longer survival time after relapse, part to differences in local aggressiveness rather than in metastatic potential, part to transient adjuvant treatment effects.
The second point relates to the definition of cutt-off points. Our results show that prognostic information may get lost if only the traditional cut-off points of positivity and negativity are used. In treating the ER as a continuous variable it was found that patients with very high levels may have a poorer prognosis. Numbers did not permit to analyze these data further regarding the relation with for instance age and MPA treatment effect. However, if this finding is true it may clearly have contributed to the inconsistency of findings in studies using traditional cut-off points.
Taken together our data show that the steroid-receptor status is a difficult and not altogether reliable prognosticator. Although further and even larger studies would be needed to delineate their role more precisely, however, it can be doubted whether they will even be of major importance in this respect. Their main value may lay in the predictive power for the chance to obtain a positive treatment effect with adjuvant endocrine therapy.

FCM IN NODE-NEGATIVE PATIENTS

The results of DNA flow cytometry (FCM) in node-negative breast cancer patients are described in chapter IV. Aneuploidy, but not SPF is an independent prognostic factor in multivariate analysis, however, the contribution of ploidy to the determination of the chance for relapse and death is too small to justify its application in routine practice. In contrast to Sigurdsson et al.[16] we did not find SPF to be an independent prognosticator. This is at least partly caused by the high number of missing SPF in our population. This

group has a favourable prognosis and if analyzed by a computer model, it would likely have been classified as to belong to the low SPF group. Sigurdsson et al. used a computer program and were able to determine SPF in 90% (rather than 60% as in our study) of all cases. If this information is taken into account, the discrepancy between the two studies disappears. A major conclusion from this chapter, therefore, can be, that what one finds regarding the relation between SPF and prognosis depends at least in part on a technical aspect: what has been done with the DNA histograms showing an overlapping aneuploid population of relatively small size. Given this dependency on technical features, the reliability of SPF measurement with this computer-based methodology for breast cancer patients can seriously be questioned.
This is not to say, that proliferation may not be important for the prognosis in breast cancer. Thymidine incorporation studies clearly show this to be the case[17,18] but are laborious. New technologies including the measurement of S-phase associated markers such as PCNA[19] and the restriction of measurements to tumour cells using a combination of proliferation and cellular markers[20] should lead to a better understanding of the relation between proliferation and prognosis.

FCM IN NODE-POSITIVE PATIENTS

Ploidy nor SPF contributed to the prognostic information already given by axillary node status, steroid-receptor status and age. In a more detailed analysis, however, ploidy predicted whether MPA added a more favourable prognosis to CAF adjuvant therapy. Patients with diploid tumours benefitted from MPA as compared to those with aneuploid tumours. Also a suggestion was obtained that in patients with a low SPF (<8%) MPA negatively influenced prognosis.
These results, although intriguing, should be viewed with caution. They refer to subgroup analyses and were not obtained in a prospective way. Furthermore, it is not easy to fit these results in our current models of breast cancer biology. They underscore, however, the potential differences between factors with a treatment-independent and treatment-dependent prognostic power, the latter often being called predictive factors. It would be worthwhile to retrospectively analyze other studies with randomized treatment allocations for the predictive effects of ploidy and proliferation. In particular, the trials with a yes or no tamoxifen arm (in addition to chemotherapy or compared to a no treatment arm) are interesting in this respect. If confirmed, our data should form the basis for prospective trials using ploidy and SPF as stratification factors.

PRIMARY TUMOUR AND LYMPH NODES

Chapter VI describes ploidy and S-phase fraction (SPF) of primary breast cancers and their nodal metastases. This study revealed that differences in DNA ploidy between primary breast cancer and their nodal metastasis can be caused by intra-tumoral heterogeneity. If clonal selection does occur during lymphogenic metastasis, this does not result in to clonal restriction.
Nodal SPF is significantly lower than the SPF in the corresponding primary tumours.

This cannot be explained by dilution effect due to admixture with non-malignant lymph node cells with low proliferation state. Apparently, the lymph node environment is more favourable to suppress proliferation as the breast tissue itself. This may be due to the presence of stroma-derived stimulators of proliferation in the breast or the active inhibition of proliferation by lymph node derived factors.

In chapter IV and V, but also in chapter VI, calculations of SPF were not aided by computer programs. From our data and from data in the literature, it is clear that sampling variations by far exceed the variations introduced by various methods of calculating SPF in a given histogram. Computer-aided S-phase analysis has the advantage to obtain a SPF in a higher percentage of histograms but not necessarily of greater accuracy.
We conclude from this chapter, that there is no reason to include lymph node FCM in the work-up of patients with node-positive breast cancer.

FINAL REMARKS

The results of all investigated variables in node-positive breast cancer patients reveals that the number of tumour containing axillary lymph nodes remains the most important prognosticator. ER is a time dependent predictor for DMFS and OS and PgR predicts for DMFS and OS. However, with the prognostic factors we studied, it is impossible to identify reliably the node-positive women with a favourable prognosis for whom adjuvant chemotherapy is not indicated or to predict which group of node-positive patients will not benefit from adjuvant treatment. More reliable prognostic factors are needed to develop strategies to predict which 30% of node-positive breast cancer patients will remain disease-free and which 30% of node-negative patients are destined to have recurrent disease and which of those may benefit from adjuvant therapies.

Many new prognostic indicators have been described over the past several years and studies are currently ongoing to assess whether any of these indicators may independently predict the likelihood of relapse. This includes the thymidine labelling index[21-23], expression of growth factor receptors such as the protein product of the HER 2/Neu oncogene[24] which is structurally homologous to the EGF-R (epidermal growth factor receptor)[25,26], the expression of insulin-like growth factor-1[27], cathepsin D production[28], and the expression of the antimitotic gene nm23[29]. Additionally, increasing evidence ascribes an independent predictive and prognostic role to alterations of the p53 tumour-suppressor gene[30] and microvessel density[31]. These factors may be helpful in the future to identify appropriate anticancer therapy.

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SAMENVATTING

SAMENVATTING

Mammacarcinoom is een heterogene en complexe aandoening waarvoor in de nabije toekomst geen dramatische verbetering in de overleving te verwachten is. Het meest tot de verbeelding sprekend resultaat is bereikt door middel van adjuvant chemotherapie bij premenopausale patiënten en langdurig adjuvant tamoxifen-behandeling bij postmenopausale patiënten.
Het laatste decennium is gekenmerkt door bestudering van een groot aantal biologische factoren, die van invloed zouden kunnen zijn op de ontwikkeling van normaal tot abnormaal mamma-weefsel en op metastaseringsprocessen van tumorcellen. Hoewel op dit moment een 60-tal variabelen onderzocht zijn, wordt de keuze van behandeling bij patiënten met een mammacarcinoom vooralsnog empirisch bepaald en slechts in geringe mate beïnvloed door biologische factoren.

In dit onderzoek bij klier-negatieve en klier-positieve mammacarcinoompatiënten, waarbij diagnose stadiëring en locoregionale therapie uniform werden toegepast, hebben wij getracht klinische en biologische kenmerken en aspecten van behandeling te relateren aan prognose en respons op de behandeling.

In hoofdstuk II zijn de klinische resultaten beschreven van postoperatieve chemo-hormonale therapie vergeleken met chemotherapie alleen bij klier-positieve borstkankerpatiënten. Patiënten ≥ 60 jaar toonden een significant betere overleving indien medroxyprogesteron acetaat (MPA) werd toegevoegd aan de chemotherapie. Dit verschil ten gunste van de gecombineerde chemo-hormonale behandeling is mogelijk te verklaren op basis van hogere oestrogeen receptorwaarden bij oudere patiënten. Bij patiënten ≤ 40 jaar werd een nadelig effect gezien bij de combinatie van chemotherapie en medroxyprogesteron acetaat (MPA) hetgeen zeer waarschijnlijk te verklaren is op basis van het achterwege blijven van door chemotherapie geïnduceerde ovariële ablatie bij toediening van hoge dosis progestagenen.

De hoge dosis MPA had een aantal nadelige effecten, namelijk gewichtstoename en abcessen als gevolg van intramusculaire toediening van MPA. Aangezien lage dosis MPA (300 mg p.o.) hetzelfde remissiepercentage heeft in vergelijking met hoge dosis MPA (900 mg p.o.) zou in de adjuvantsetting een lage orale dosis gebruikt kunnen worden om deze bijwerkingen te voorkomen. Aangezien hoge dosis MPA even effectief

is als tamoxifen in het gemetastaseerd mammacarcinoom verdient onderzoek met lage dosis MPA (300 mg p.o.) als adjuvant behandeling bij postmenopausale lymfklierpositieve mammacarcinoompatiënten een plaats. Men zou kunnen denken aan een klinisch vergelijkend onderzoek waarbij bovengenoemde MPA-dosering vergeleken wordt met de standaard-behandeling tamoxifen 20 mg. Tamoxifen 20 mg. toegediend gedurende een periode van 2 à 3 jaar heeft interessante neveneffecten zoals voorkoming van osteoporose en cardiovasculaire complicaties. Eveneens verminderd tamoxifen de incidentie van contralateraal mammacarcinoom, echter verhoogd mogelijk het risico op endometriumcarcinoom. Dit laatst genoemd probleem treedt niet op bij medoxyprogesteron acetaat en wat betreft het contralateraal mammacarcinoom heeft onderzoek, verricht door onze groep, aangetoond dat MPA even effectief is als tamoxifen voor wat betreft preventie van contralateraal mammacarcinoom.

Concluderend kan gesteld worden dat het momenteel nog te vroeg is om op grote schaal anti-oestrogenen te vervangen door progestagenen bij de adjuvant behandeling van klier-positieve postmenopausale mammacarcinoom patiënten. Onderzoeken waarbij langdurig tamoxifen gebruik vergeleken wordt met kortdurend en langdurend oraal progestageengebruik zijn noodzakelijk om effectiviteit en bijwerkingen van deze middelen te vergelijken.

In hoofdstuk III worden steroïdreceptoren als prognostische factoren bij het mammacarcinoom bestudeerd. In de literatuur wordt als afkappunt voor de steroïdreceptoren meestal 10 fmol/mg eiwit gehanteerd. In hoofdstuk III is de oestrogeen en de progesteronreceptor als een continue variabele bestudeerd en is voor elke waarde het relatieve risico bepaald.
Klier-negatieve mammacarcinoompatiënten met oestrogeenreceptorwaarden tussen de 100 en 400 fmol/mg eiwit hebben een significant lager risico in vergelijking met patiënten met oestrogeenreceptorwaarden <100 of >400. Patiënte met zeer hoge ER-waarden (>400 fmol/mg) hadden een duidelijk slechtere prognose, echter dit fenomeen zou beïnvloed kunnen zijn door het lage aantal patiënten in deze groep.
In de klier-positieve patiënten is een afkapwaarde van 10 fmol/mg eiwit een tijdsafhankelijke prognostische factor, dat wil zeggen dat een oestrogeenreceptorwaarde van ≤ 10 fmol/mg gedurende de eerste 5 jaren na de diagnose een prognostisch ongunstige betekenis heeft.
De progesteronreceptor is een tijdsafhankelijke prognostische factor bij klier-negatieve patiënten. Patiënten met een progesteronwaarde ≤ 80 fmol/mg eiwit hebben de eerste 5 jaren na de diagnose een significant hoger risico op metastasen op afstand en een hoger risico om te overlijden als gevolg van het mammacarcinoom in vergelijking met patiënten die een progesteronreceptorwaarde hebben die >80 fmol/mg eiwit bedraagt. In de klier-positieve patiënten wordt een afkappunt voor de progesteronreceptorwaarde gezien die licht verschilt ten opzichte van de klier-negatieve patiënten (60 fmol/mg) en wordt bij een waarde van ≤ 60 een significant hoger risico waargenomen. Dit effect is onafhankelijk van de follow-up duur.

Deze gegevens en de literatuurgegevens laten geen gefundeerde conclusies toe omtrent het gebruik van de steroïdreceptor als prognostische factor voor patiënten met een

resectabel mammacarcinoom. Twee facetten in onze studie zijn opvallend. Op de eerste plaats de tijdsafhankelijkheid van de prognostische factor. In onze studie is een lage oestrogeenreceptor waarde bij klier-positieve patiënten en een lage progesteronreceptor waarde bij klier-negatieve patiënten een prognostisch ongunstige factor gedurende de eerste 5 jaren na de diagnose. Dit effect verdwijnt na een follow-up periode van 5 jaar. Het is niet geheel duidelijk wat de oorzaak is van dit fenomeen; mogelijk moeten we de oorzaak zoeken in een langere overleving vanaf het moment van recidief bij patiënten met een steroïdreceptor positieve tumor, mogelijk dat het verschil veroorzaakt wordt door een voorbijgaand effect van de adjuvante therapie. Het tweede aspect dat in onze studie naar voren toe komt is de bepaling van de afkappunten van de steroïdreceptorwaarden. Onze resultaten laten zien dat de prognostische betekenis van de steroïdreceptor verdwijnt indien traditionele afkappunten worden gehanteerd. Beschouwen we de oestrogeenreceptor als een continue variabele dan bleek in ons onderzoek dat patiënten met een zeer hoge waarde een ongunstige prognose hadden. Gezien het lage aantal patiënten in deze groep was het niet mogelijk om een relatie te leggen met bijvoorbeeld de leeftijd en het MPA-effect in deze prognostisch ongunstige groep.
Concluderend kan gesteld worden dat de steroïdreceptorstatus een moeilijk te beoordelen een niet al te betrouwbare prognostische factor is. Zeer waarschijnlijk ligt de waarde meer in de predictieve kracht om een positief behandelingsresultaat te voorspellen bij adjuvante endocriene therapie.

In hoofdstuk IV worden de resultaten beschreven van DNA-flow cytometrie (FCM) bij klier-negatieve mammacarcinoom patiënten. Aneuploidie, echter niet SPF, is een onafhankelijke prognostische factor in de multivariate analyse. Echter de prognostische waarde van aneuploïdie is niet hoog genoeg om routine-matig toe te passen bij patiënten met een mammacarcinoom.
In tegenstelling tot een aantal publikaties bleek in ons onderzoek dat SPF geen onafhankelijke prognostische factor was. Dit wordt gedeeltelijk veroorzaakt doordat in een hoog percentage van de DNA-histrogrammen de SPF-bepaling niet mogelijk was. Deze groep heeft in ons onderzoek een gunstige prognose en dienen hoogst waarschijnlijk in de lage SPF-groep te worden geclassificeerd indien gebruik gemaakt zou zijn van een computermodel om alsnog SPF te bepalen.
Na onze mening dient de conclusie van dit hoofdstuk te zijn dat de correlatie tussen SPF en prognose afhankelijk is van de procedure die gevolgd is om DNA-histrogrammen met overlappende aneuploïde populaties of relatief kleine aneuploïde populaties te beoordelen. In onze ogen is de betrouwbaarheid van de SPF-bepaling door middel van computermodellen voor het mammacarcinoom discutabel.
Dit wil niet zeggen dat bepaling van het percentage prolifererende cellen niet van belang zou zijn bij de prognose bij het mammacarcinoom. Onderzoeken met thymidine-incorporatie hebben aangetoond dat dit zeker het geval is, echter deze methode is zeer bewerkelijk en tijdrovend. Nieuwe technieken om het aantal prolifererende cellen te bepalen zoals de PCNA-techniek en het bepalen van SPF op alleen de tumorcelpopulaties, gebruik makend van proliferatie en cellulaire makers kunnen meer inzicht geven in de relatie tussen proliferatie-prognose.

In hoofdstuk V worden de resultaten van flow cytometrie in klier-positieve patiënten beschreven. Uit ons onderzoek blijkt dat ploïdy noch SPF bijdragen aan het bepalen van de prognostische betekenis bij klier-positieve patiënten. In een meer gedetailleerde analyse bleek dat diploïde patiënten een gunstiger prognose hadden ten opzichte van aneuploïde patiënten indien in de adjuvante behandeling progestagenen aan de chemotherapie werden toegevoegd. Eveneens was opvallend dat bij de patiëntengroep met een lage SPF-waarde (<8%) toevoeging van MPA een negatieve invloed had op de prognose.
Hoewel deze resultaten interessant zijn dienen we bij de interpretatie hiervan voorzichtigheid te betrachten. Het betreft hier een sub-groepanalyse en de resultaten zijn retrospectief onderzocht. De resultaten onderstrepen wel dat we rekening dienen te houden met factoren die behandelings afhankelijk (predictieve factoren) en behandelings onafhankelijk zijn.
Gezien het vastgestelde resultaat zou het interessant zijn andere studies met eenzelfde randomisatie retrospectief te analyseren voor wat betreft de predictieve effecten van ploïdy en proliferatie. Vooral de onderzoeken waarbij al of niet tamoxifen werd toegevoegd aan chemotherapie in de adjuvant-setting zijn interessant wat betreft dit aspect. Indien onze bevindingen worden bevestigd zou dit een prospectief onderzoek rechtvaardigen wat bij ploïdy en SPF als stratificatie-factoren zullen worden onderzocht.

Hoofdstuk VI vergelijkt ploïdy en SPF in primaire mammacarcinomen en nodale metastasen. Dit onderzoek laat zien dat verschillen in DNA-ploïdy tussen de primaire tumor en de nodale metastase veroorzaakt worden door intratumorale heterogeniteit. Het percentage S-fase cellen was in tumorcellen gemetastaseerd naar axillaire klieren significant lager dan de corresponderende primaire tumor. Ons inziens kan dit niet worden verklaard op grond van een daling van de SPF als gevolg van vermenging met lymfoïde cellen met een lage proliferatiefractie. Mogelijk dat er een proliferatieremmende factor uitgaat van de lymfatische cellen; mogelijk dat de afwezigheid van stroma-afhankelijke stimulatoren voor proliferatie een verklaring zijn voor dit fenomeen.

In hoofdstuk IV en V, maar ook in hoofdstuk VI werden SPF-berekeningen niet verricht met behulp van computermodellen. Uit onze data en ook uit de data van de literatuur is bekend dat variaties als gevolg van de plaats waar het biopt genomen is (samplingvariaties) veel belangrijker zijn dan de variaties veroorzaakt door de verschillende methoden om SPF-waarden te berekenen. Naar onze mening is het met behulp van computermodellen mogelijk om een hoger percentage SPF-waarde te bepalen, echter betekent dit niet dat hiermee ook de nauwkeurigheid wordt verhoogd.
Uit dit hoofdstuk kan geconcludeerd worden dat er geen noodzaak bestaat om DNA-flow cytometrie te verrichten van axillaire lymfeklieren bij patiënten met een klier-positief mammacarcinoom.

Eindconclusie.
Indien we alle onderzochte variabelen bij klier-positieve mammacarcinoom patiënten kritisch beschouwen blijkt dat het aantal tumor bevattende axillaire lymfeklieren de meest belangrijke prognostische factor blijft. ER is een tijdsafhankelijke predictor voor DMFS

en OS en de PgR heeft een predictieve waarde voor wat betreft de DMFS en OS. Echter met de prognostische factoren die we hebben bestudeerd is het vooralsnog niet mogelijk om klier-positieve patiënten met een gunstige prognose voor wie adjuvant chemotherapie niet is geïndiceerd of anderzijds de groep van klier-positieve patiënten die geen gunstig effect hebben van de adjuvant therapie te identificeren. We hebben behoefte aan een aantal betrouwbare prognostische factoren om de groep van 30% van de klier-positieve mammacarcinomen die ziektevrij blijven en de groep van 30% van de klier-negatieve patiënten die zullen recidiveren te identificeren.

Een groot aantal nieuwe variabelen zijn de laatste jaren onderzocht en worden momenteel onderzocht naar de prognostische betekenis. Naast proliferatiefactoren wordt momenteel onderzoek verricht naar factoren die van belang zijn bij geprogrammeerde celdood (apoptosis) en worden de verschillende factoren die van belang zijn bij het proces van metastasering onderzocht. Deze factoren kunnen van belang zijn om in de toekomst de behandeling bij het mammacarcinoom beter af te stemmen.

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PUBLICATIONS

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CURRICULUM VITAE

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De auteur van dit proefschrift is in de periode 1972-1975 praktiserend huisarts geweest te Geleen, in samenwerking met Drs P Mekel. Na een periode werkzaam te zijn geweest in het Dr A Mathijssen Ziekenhuis te Utrecht (Dr M van Zoeren, Dr A van Belle) en in het St.Joseph Ziekenhuis te Kerkrade (Drs J Scherpbier), begon hij de opleiding interne geneeskunde in het Annadal Ziekenhuis te Maastricht (1976-1978 opleider Drs J Coenegracht). Deze opleiding werd voltooid bij Prof Dr C Majoor, hoofd en opleider van de Afdeling Interne Geneeskunde aan de Katholieke Universiteit te Nijmegen.
Verdere bekwaming in de Haematologie (Prof Dr C Haanen) en de oncologie (Prof Dr DJTh Wagener) vond plaats aan voornoemde universiteit.
Sedert mei 1983 is hij verbonden aan de Afdeling Interne Geneeskunde van het Academisch Ziekenhuis te Maastricht (hoofd: Prof Dr JA Flendrig).